Universidade de Aveiro Departamento de Química
2017

Sofia Maria Soares da Costa

Impacto cardiovascular da citotoxicidade e do envelhecimento na agregação de proteínas

Cardiovascular impact of cytotoxicity and aging in protein aggregation

Universidade de Aveiro Departamento de Química
2017

Sofia Maria Soares da Costa

Impacto cardiovascular da citotoxicidade e do envelhecimento na agregação de proteínas

Cardiovascular impact of cytotoxicity and aging in protein aggregation

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Mestre Dulce Fontoura, Mestre em Fisiopatologia Cardiovascular e Especialista pela Universidade de Aveiro, e co-orientação da Professora Doutora Inês Falcão Pires, Professora Auxiliar da Faculdade de Medicina da Universidade do Porto e da Professora Doutora Rita Ferreira, Professora Auxiliar do Departamento de Química da Universidade de Aveiro.

o júri

presidente

Prof. Doutor Manuel António Coimbra Rodrigues da Silva
Professor associado com agregação, Universidade de Aveiro

Mestre Dulce Marlene Martins Fontoura
Mestre em Fisiopatologia Cardiovascular, Especialista, Faculdade de Medicina da Universidade do Porto

Doutor José Pedro Quinta Araújo Castro
Investigador de Pós Doutoramento, German Institute of Human Nutrition Potsdam-Rehbrücke

agradecimentos

No final de mais uma etapa académica resta-me agradecer a todos os que de certa forma contribuíram para a realização deste trabalho.

Ao Professor Doutor Adelino Leite Moreira, Diretor do Departamento de Fisiologia e Cirurgia Cardiotorácica do Centro de Investigação Médica da Faculdade de Medicina da Universidade do Porto, agradeço pela oportunidade de realizar a minha tese de mestrado nesta instituição.

À Professora Doutora Inês Falcão Pires agradeço pela oportunidade e pelo privilégio em ter feito parte da sua equipa de investigação. Muito obrigada pelo tempo despendido durante este ano bem como por todas as correções e sugestões para melhorar este trabalho.

À minha orientadora, Dulce Fontoura, Mestre em Fisiopatologia Cardiovascular, agradeço por me ter recebido tão bem e por me ter acompanhado de forma presente e dedicada no decorrer deste projeto. Muito obrigada por todo o apoio, compreensão, encorajamento e disponibilidade sempre demonstrada durante este ano. Espero ter sido capaz de corresponder a todas as expetativas.

Aos Professores Doutores Rui Vitorino e António Barros agradeço pela colaboração e por todos os ensinamentos que muito contribuíram para o enriquecimento deste trabalho.

À Professora Doutora Rita Ferreira agradeço pelo entusiasmo, pelas sugestões e pela disponibilidade sempre demonstrada para esclarecer qualquer dúvida.

Não podia deixar de agradecer ao Fábio Trindade por toda ajuda e disponibilidade. Sem dúvida que os teus conhecimentos científicos foram imprescindíveis para a realização deste trabalho. É sempre bom trabalhar com pessoas tão profissionais e ao mesmo tempo tão prestáveis.

Agradeço também à Daniela Miranda, Cláudia Mendes e Glória Almeida pela simpatia com que me receberam, por todas as gargalhadas e ensinamentos ao longo deste ano.

À Patrícia Rodrigues, um agradecimento muito especial por todo o apoio desde o início. Obrigada pela tua amizade, pela motivação e por todos os conselhos nas horas mais difíceis. Obrigada por estares sempre disponível para me esclarecer todas as dúvidas e pela tua dedicação a esta tese. Aprendi muito contigo. Gosto muito de ti Tixa.

À Tânia Lima e à Raquel Videira obrigada por serem o meu apoio durante este ano. Obrigada pela amizade sincera, por ouvirem os meus desabafos e acima de tudo pelos bons momentos. Gosto muito de vocês miúdas.

Aos meus amigos do coração obrigada por estarem sempre presentes. Ao João, por estares sempre comigo e pelo apoio incondicional. Por fim, um agradecimento muito especial a toda a minha família. Em particular, à minha irmã por me aturar sempre. Ao meu irmão por relativizar as coisas. Aos meus pais, às pessoas mais importantes da minha vida, obrigada por tornarem tudo isto possível, por terem acreditado sempre em mim e por me terem ensinado a nunca desistir. A vocês dedico este trabalho.

palavras-chave

Agregação proteica, sistemas de controlo de qualidade proteica, envelhecimento, doxorrubicina, cardiotoxicidade, doenças cardiovasculares.

resumo

A estrutura nativa das proteínas, decorrente do *folding* proteico, constitui um pré-requisito para a sua funcionalidade. No entanto, vários fatores promovem o *folding* incorreto (*misfolding*) de proteínas, causando a sua agregação. Recentemente, o *misfolding* e a agregação de proteínas têm sido associados às doenças cardiovasculares, que representam a principal causa de morte em todo o mundo. De forma a impedir a formação de agregados proteicos potencialmente tóxicos, os cardiomiócitos desenvolveram sistemas de controlo de qualidade proteica. Contudo, o seu comprometimento potencia a acumulação de proteínas disfuncionais na forma de agregados. Neste contexto, e tendo em consideração o papel do envelhecimento e da doxorrubicina (Doxo) no aumento do risco para o desenvolvimento de doenças cardiovasculares, será importante esclarecer a associação entre cada um destes fatores de risco e a agregação proteica. Deste modo, procuramos otimizar uma metodologia de enriquecimento de agregados proteicos provenientes de ventrículo esquerdo (VE) de um modelo animal de envelhecimento e de um modelo animal de cardiotoxicidade induzida pela Doxo, visando identificar as proteínas presentes nestes agregados por GeLC-MS/MS. A técnica de isolamento de frações enriquecidas em agregados proteicos foi otimizada com sucesso em ambos os modelos animais. A análise por GeLC-MS/MS permitiu a identificação de 1279 e 1260 proteínas no VE de ratos WKY novos e velhos, respetivamente. A análise diferencial revelou que 15 e 18 proteínas apresentavam níveis mais elevados nos agregados do grupo novos e velhos, respetivamente. Entre as proteínas presentes em maior quantidade nos ratos envelhecidos destacam-se aquelas relacionadas com a contração cardíaca (miosina-6 e miosina-7), o *folding* mediado pelas chaperonas (TRiC) e sistemas proteolíticos (catepsina D). Em relação ao modelo animal de cardiotoxicidade, 274 proteínas foram identificadas no grupo controlo e 267 no grupo Doxo. A análise diferencial revelou que apenas uma proteína, a glicoproteína rica em histidina (fragmento), apresentava níveis mais elevados nos agregados de animais tratados com Doxo. Esta proteína está envolvida na regulação de vários processos biológicos, como a inflamação e a angiogénese, sugerindo um possível papel da mesma na cardiotoxicidade induzida pela Doxo. A identificação destas proteínas bem como o conhecimento da sua relevância biológica fornece informações valiosas sobre o comprometimento da homeostasia das proteínas, tanto no envelhecimento como em condições de cardiotoxicidade. Desta forma, estudos futuros serão necessários para elucidar sobre o impacto real da agregação destas proteínas no envelhecimento cardíaco e em condições de cardiotoxicidade, bem como potenciais alvos terapêuticos.

keywords Protein aggregation, protein quality control systems, aging, doxorubicin, cardiotoxicity, cardiovascular diseases.

abstract Native structure of proteins, acquired by protein folding, is required for them to function properly. However, several factors promote incorrect protein folding (misfolding), causing their aggregation. Recently, protein misfolding and aggregation have been associated with cardiovascular diseases, the leading cause of death worldwide. In order to avoid the generation of potentially toxic protein aggregates, cardiomyocytes have developed protein quality control systems. However, failure of these systems promotes the accumulation of abnormal protein aggregates. In this context, and taking into account the burden of aging and doxorubicin (Doxo) for cardiovascular diseases progression, it will be important to clarify the association between these two risk factors and protein aggregation. Therefore, we aimed to optimize the methodology for protein aggregates enrichment from left ventricle (LV) of aging and Doxo-induced cardiotoxicity animal models, and also to identify the proteins presented in these aggregates by GeLC-MS/MS. In both animal models, the technique for isolation of protein aggregates-enriched fractions was successfully optimized. GeLC-MS/MS analysis allowed the identification of 1279 and 1260 proteins in young and aged WKY LV, respectively. Differential protein analysis revealed that 15 and 18 proteins were presented at higher amounts in young and aged groups, respectively. Among proteins with greater amounts in aged rats, we highlighted those related to cardiac contraction (myosin-6 and myosin-7), chaperone-mediated protein folding (TRiC) and proteolytic systems (cathepsin D). Regarding the animal model of cardiotoxicity, 274 proteins were identified in the control group and 267 in the Doxo group. Differential protein analysis revealed that only one protein, histidine-rich glycoprotein (fragment), was presented in higher amounts in aggregates from Doxo-treated animals. This protein is involved in the regulation of several biological processes, such as inflammation and angiogenesis, suggesting that it can play a role in Doxo-induced cardiotoxicity. The identification of these proteins as well as the knowledge of their biological relevance provides valuable information about protein homeostasis impairment, both in aging and in cardiotoxicity conditions. Therefore, future studies are necessary to elucidate the real impact of the aggregation of these proteins on cardiac aging and cardiotoxicity conditions, as well as potential therapeutic targets.

ABBREVIATIONS:

ACN: Acetonitrile
ADP: Adenosine Diphosphate
Akt (or PKB): Protein Kinase B
AMP: Adenosine Monophosphate.
AMPK: AMP-Activated Protein Kinase
ATF6: Activating Transcription Factor 6
Atg: Autophagy-Related Genes
ATP: Adenosine Triphosphate
Bcl-2: B-cell lymphoma 2
DNA: Deoxyribonucleic Acid
Doxo: Doxorubicin
DTT: Dithiothreitol
E1: Ubiquitin-activating enzyme
E2: Ubiquitin-conjugating enzyme
E3: Ubiquitin ligase
EF: Ejection Fraction
Erk1/2: Extracellular Signal-Regulated Kinase 1/2
FDR: False Discovery Rate
GeLC-MS/MS: SDS-PAGE followed by Liquid Chromatography-Tandem Mass Spectrometry
GRAVY: Grand Average of Hydropathy
GRP78: Glucose-Regulated Protein 78
HF: Heart Failure
HRG: Histidine-Rich Glycoprotein
HRR: Histidine-Rich Region
HSP: Heat Shock Proteins
IAA: Iodoacetamide
IL-1: Interleukin-1
IRE1: Inositol-Requiring Enzyme-1
LAMP: Lysosome-Associated Membrane Protein
LC3: Microtubule-Associated Protein Light Chain 3
LV: Left Ventricle
MAFbx: Muscle Atrophy F-box
MAO: Monoamine Oxidase
MAPK: Mitogen-Activated Protein Kinase
MS: Mass Spectrometry
mTOR: Mammalian Target of Rapamycin
MURF-1: Muscle RING-finger protein-1
MVP: Major Vault Protein
MyBPC: Myosin Binding Protein C
Na^+/K^+-ATPase: Sodium/Potassium-transporting ATPase
NBR1: Neighbor of BRCA1 Gene 1
NEF: Nucleotide-Exchange Factors
NIH: National Institutes of Health
P70S6K: Ribosomal Protein S6 Kinase Beta-1
PE: Phosphatidylethanolamine
PERK: Protein Kinase RNA-like Endoplasmic Reticulum Kinase
PGC-1α: Peroxisome proliferator-activated receptor-gamma coactivator
PI3K: Phosphatidylinositol 3-Kinase
PINK1: PTEN-Induced Putative Kinase 1
PRR: Proline-Rich Regions
RNA: Ribonucleic Acid

ROS: Reactive Oxygen Species
RyR: Ryanodine Receptor
Sarcosyl: N-lauroylsarcosinate
SDS: Sodium Dodecyl Sulphate
SDS-PAGE: SDS Polyacrylamide Gel Electrophoresis
SERCA: Sarcoplasmic Reticulum Ca^{2+}-ATPase
sHSP: Small Heat Shock Proteins
SR: Sarcoplasmic Reticulum
TCA: Trichloroacetic Acid
TFEB: Transcription Factor EB
TRiC: T-complex protein-1 ring complex
ULK1: UNC-51 like kinase 1
UPS: Ubiquitin-Proteasome System
WKY: Wistar Kyoto

INDEX

1. INTRODUCTION 2

1.1 Protein misfolding and aggregation 4
1.2 Protein quality control systems 5
1.2.1 Chaperones 5
1.2.2 Ubiquitin-proteasome system 7
1.2.3 Autophagy 9
1.3 Dysregulation of proteostasis in aging 12
1.4 Dysregulation of proteostasis caused by doxorubicin 15
1.5 Methodological approaches to study protein aggregates 19

2. AIMS 20

3. MATERIALS AND METHODS 21

3.1 Animal model 21
3.2 Baseline protocol for protein aggregates extraction and purification 21
3.3 In-gel protein digestion 22
3.4 Protein identification by GeLC-MS/MS 22
3.5 Bioinformatic and Statistical analysis 23

4. RESULTS 24

4.1 Methodological optimization for the isolation and characterization of the protein aggregates-enriched fractions 24
4.2 Optimized protocol for the isolation of protein aggregates-enriched fractions 30
4.3 Aging animal model 31
4.3.1 Characterization of the protein aggregates-enriched fractions isolated from left ventricle of young and aged rats 31
4.3.2 Proteomic analysis after GeLC-MS/MS 32
Total protein aggregates-enriched fractions 32
Hydropathy analysis 33
Biological processes 33
Differential protein analysis 35
Human diseases associated with aggregated proteins 37
4.4 Doxo-induced cardiotoxicity animal model 39
4.4.1 Characterization of the protein aggregates-enriched fractions isolated from left ventricle of Control and Doxo-treated rabbits 39
4.4.2 Proteomic analysis after GeLC-MS/MS 40
Total protein aggregates-enriched fractions 40
Hydropathy analysis 41
Biological processes 41
Differential protein analysis 42
Human diseases associated with aggregated proteins 43

5. DISCUSSION 45

5.1 Methodological optimization for the isolation and characterization of the protein aggregates-enriched fractions 45
5.2 Proteomic analysis of protein aggregates-enriched fractions 47
Cardiovascular impact of aging in protein aggregation 47
Cardiovascular impact of Doxo-induced cardiotoxicity in protein aggregation 52

6. CONCLUSIONS AND FUTURE PERSPECTIVES 56

7. REFERENCES 57

1. INTRODUCTION

The process by which proteins acquire their native structure, named as folding, is crucial for proteins to perform their functions in cell and, consequently, essential to all biological processes. On the other hand, unfolding or incorrect folding (misfolding) of proteins and consequent loss of function may have catastrophic effects for cells functioning and trigger protein aggregation, which is *per se* toxic for the cell (1, 2). At the protein native structure, hydrophobic residues are mainly found in their core. However, when protein misfolds, a structural change exposes the hydrophobic residues, allowing them to interact in a non-specific way with other proteins causing abnormal protein aggregation (1). Moreover, it is known that excessive accumulation of misfolded proteins is related to the manifestation or progression of several cardiovascular diseases (2, 3). Therefore, the existence of protein quality control systems that ensure the correct synthesis and folding of proteins as well as the identification and repair or degradation of proteins with abnormal conformation are crucial for cells survival, namely cardiomyocytes (2). The systems responsible for protein quality control, which include chaperones and proteolytic systems, are essential for proteostasis, which means, the maintenance of proteome homeostasis.

Chaperones are specialized proteins that interact, stabilize or assist other proteins to acquire their functionally active conformation, being absent in the final structure. Beyond participating in proteins folding, these molecules recognize and repair misfolded proteins, by binding to hydrophobic regions, thereby preventing aggregation. Additionally, chaperones are involved in protein transport, assembly of oligomeric proteins and proteolytic degradation (4). However, if chaperone activity becomes compromised, other systems will be initiated in order to restore proteostasis (2). Thus, sarcoplasmic reticulum (SR)-associated protein degradation, proteasomes, calpains system and lysosomal and mitochondrial proteolytic enzymes will work together in recycling or removing protein aggregates at a cellular level (5). Between proteolytic systems, ubiquitin-proteasome system (UPS) and autophagy are the two main pathways for protein degradation.

UPS represent the second line of defense, being responsible for the degradation of misfolded, mutated or any other damaged soluble proteins. Moreover, UPS is responsible for the degradation of normal proteins that are no longer needed by the cells, contributing to the temporal regulation of protein activity. This process involves two main steps: protein ubiquitination and the subsequent degradation mediated by proteasomes. Ubiquitination consists in a post-translational modification in which the binding of a polyubiquitin chain to the target protein occurs through a cascade of enzymatic reactions, promoting its subsequent degradation by the proteasome (6).

Lastly, autophagy allows degradation of dysfunctional organelles, misfolded proteins and protein aggregates (7). Autophagy, more specifically macroautophagy, involves the formation of a double-membrane vesicle around the substrates and its subsequent degradation through lysosomes (8, 9). Thus, chaperones and proteolytic systems ensure an optimal proteostasis and an adequate function of cardiomyocytes, both under physiological and stress conditions.

However, when protein quality control systems are compromised, pathological conditions associated with protein misfolding and aggregation become evident. In fact, abnormal intracellular or extracellular accumulation of protein aggregates has been associated with the pathogenesis of several neurodegenerative diseases, such as Alzheimer's, Huntington's and Parkinson's disease (1). The toxicity of these aggregates has been attributed to diverse factors, such as oxidative stress and mitochondrial dysfunction (10), proteasome inhibition (11) and autophagy impairment (12). Similarly, recent evidence suggests the presence of protein aggregates in cardiovascular diseases (3, 7), the leading cause of death in Europe, representing an important public health problem nowadays. It is estimated that more than 4 million of people die each year due to cardiovascular diseases. There are several risk factors associated with the development of cardiovascular diseases, including hypertension, obesity, aging, antineoplastic agents, among others (13, 14).

Indeed, aging associates to the rising of cardiovascular diseases prevalence, aggravated by the ageing of the population, mostly ascribed to the improvement of medical treatments and consequent increase in the average of life expectancy (15). As part of aging process several changes in cardiac structure and function take place, such is the case of: 1) increased formation of reactive oxygen species (ROS) and oxidative stress; 2) progressive accumulation of damages in deoxyribonucleic acid (DNA), in ribonucleic acid (RNA), in lipids and in proteins (16, 17); 3) aggregation of proteins (18); 4) impaired metabolism; 5) changes in calcium (Ca^{2+}) homeostasis (17); 6) cardiac fibrosis; 7) mitochondrial dysfunction; modifications in UPS and autophagy, mitophagy and apoptosis processes (16, 19), and 9) decreased number of cardiomyocytes compensated by hypertrophy of the remaining cardiomyocytes (20).

Oncological diseases are the second cause of death worldwide. Nowadays, there are different radioactive and chemical treatments aiming to eliminate tumor cells. Over the last few years, enhancement in antineoplastic therapies has contributed to a significant improvement in the prognosis of oncological patients and also to a reduction in mortality of this population (13). Doxorubicin (Doxo), a member of the anthracycline family, is widely used in the treatment of several types of cancers, including leukemia, lymphoma and breast cancer. Besides Doxo effectiveness in tumor treatment, this chemotherapy drug is known to induce cardiovascular side-effects, such as arrhythmia, cardiomyopathy and heart failure (HF), therefore limiting its clinical use (21-23). These cardiotoxic effects are usually progressive and irreversible, causing a negative

impact both in success of oncological treatment and in the life quality of patients (13, 23). At the molecular level, there are many mechanisms proposed to explain Doxo-induced cardiotoxicity, despite not been fully understood. Among the proposed mechanisms it is important to highlight the changes in mitochondrial function, ROS and oxidative stress increase, dysregulation of Ca^{2+} handling, myofibrillar degradation, DNA damage as well as UPS and autophagy dysfunction and apoptosis (23-26).

For all these reasons, this thesis aims to address the role of protein quality control systems in cardiac tissue, as well as the consequences of its dysregulation in the formation of protein aggregates. At the same time, it will also provide a general perspective of the impact of aging and Doxo in proteostasis dysregulation and protein aggregation associated to the progression of cardiovascular diseases.

1.1 Protein misfolding and aggregation

It is known that partially folded or misfolded proteins are prone to aggregation. Protein aggregates can adopt distinct structural organizations, such as soluble oligomers, amorphous aggregates, amyloid fibrils, inclusion bodies and aggresomes (4, 27, 28). With regard to the soluble oligomers, small aggregates composed by approximately 3-50 monomers (29), they represent intermediate species that can give rise to amorphous aggregates or amyloid fibrils (27). The way how aggregation goes depends on several factors such as temperature, pH (28) and peptide concentration (30).

Amyloid fibrils consist in very well organized β-sheet conformation structures. These insoluble aggregates are rigid, unbranched and deposited in the extracellular or intracellular medium. Up to now, several amyloidogenic proteins were identified, being the main feature of these proteins their structural instability, which can be induced by mutations, post-translational modifications or specific medium conditions (such as pH and temperature) (31). Furthermore, formation of these structures occurs in several organs, namely in the heart, which may lead to the development of many cardiac diseases, such as HF (32).

Additionally, the formation of larger protein aggregates, termed inclusion bodies, can occur. These structures resulted from coalescence of individual aggregates into a single one, and can contain amorphous aggregates and amyloid fibrils. Inclusion bodies can be transported along microtubules to a perinuclear region, where they form a structure denominated aggresome (27). Aggresome formation is thought to represent a cellular protective mechanism, because it allows the sequestration of misfolded proteins and aggregates in a single site, helping their subsequent removal by autophagy (29). However, it remains unclear which are the toxic species to the cells. In

fact, some studies demonstrate that soluble and smaller intermediate species, such as pre-amyloid oligomers, represent the major toxic threat for cells in detriment of larger aggregates, being the latter poorly correlated with the severity of clinical manifestation, for example the Alzheimer's disease (27, 33).

There are many internal and external conditions that contribute to protein aggregation, namely mutations, environmental stress factors such as temperature, aging and oxidative stress (34). The role of post-translational modifications, which can occur as a consequence of the above-mentioned conditions, on protein aggregates formation has been increasingly recognized (5). For instance, when amino acids like proline, arginine, threonine or lysine are exposed to high levels of ROS, they became more susceptible to carbonylation. In turn, protein carbonylation is associated with loss of enzymatic activity, loss of ligand binding properties, increased susceptibility to proteolytic activity, aggregation and modification in transcriptional activities (35-37). Post-translational modifications are not only involved in the formation of protein aggregates but also in dysregulation of proteolytic system components. In fact, excessive levels of ROS, like hydrogen peroxide (H_2O_2) and lipid peroxides, can inhibit or change many proteasome subunits, thereby compromising protein degradation and promoting aggregation of misfolded protein (5, 38, 39).

1.2 Protein quality control systems

1.2.1 Chaperones

Heart is constantly under stress, even under physiological conditions, thereby requiring a continuous recycling and maintenance of proteins in order to work properly (2). For this reason, cardiomyocytes have developed quality control systems to establish a perfect balance between protein synthesis and degradation. In first place, chaperones play a crucial role in protein homeostasis (40), being present in cytosol, nucleus, mitochondria and SR of cardiomyocyte (41). Besides their participation in protein folding, chaperones regulate protein synthesis, transport, aggregation, disaggregation and degradation (4). They are also involved in intracellular signaling, controlling the conformational changes necessary to activation or deactivation of signaling mediators and their binding to signalosomes (42), a complex intracellular network composed of several components of different signaling pathways (43).

Chaperones are proteins constitutively expressed in cardiomyocytes. Nevertheless, their expression increases under stress conditions in order to maintain protein homeostasis and prevent protein aggregates formation. Insoluble aggregates of non-sarcomeric or sarcomeric misfolded proteins are toxic to the cardiac cell, and may even trigger apoptosis. Increased chaperones

expression has as main goal to avoid accumulation of these misfolded proteins, by promoting their refolding or directing them to be degraded by UPS or autophagy (40).

The chaperone machinery is composed by numerous proteins that cooperate with each other, and are typically known as stress proteins or heat shock proteins (HSP), once its expression is increased under stress conditions. In cardiomyocytes, they are usually divided into three main classes: the general chaperones (HSP70 and HSP90), the small heat shock proteins (sHSP) and the SR chaperones (40). Proteins that belong to HSP70 and HSP90 class, which have an ATPase domain, recognize and bind to exposed hydrophobic regions of the non-native conformation proteins, promoting their folding or refolding through several cycles of ATP binding and hydrolysis (4). HSP70 and HSP90 interact with other proteins, the co-chaperones, which support chaperones to perform their functions, including protein folding (44). For example, the HSP70 reaction cycle is controlled not only by co-chaperone HSP40 but also by nucleotide-exchange factors (NEF). The hydrolysis of ATP into ADP is strongly accelerated by HSP40, thereby triggering the binding between substrate and HSP70. Moreover, HSP40 is capable of directly binding to unfolded polypeptides and recruit HSP70. After ATP hydrolysis, the binding of NEF to HSP70 ATPase domain triggers the catalysis of ADP-ATP exchange, resulting in substrate release. Accordingly, the substrate release allows fast-folding molecules to hide hydrophobic residues, avoiding their aggregation (Figure 1). Proteins unable to acquire their native structure after HSP70 cycle, such as actin and tubulin, can be redirected to chaperonins (HSP60 class). Chaperonins exhibit a structure capable of attaching a single unfolded protein at a time, allowing more stable conditions for a proper protein folding (4, 45). Regarding HSP90, this chaperone has been associated with several important signaling pathways in eukaryotic cells, including cell cycle, telomere maintenance, apoptosis, mitotic signal transduction, vesicle-mediated transport, innate immunity and target protein degradation (4). The evolution and maintenance of these functional networks is thought to depend on the ability of HSP90 to attenuate the effects of structurally destabilizing mutations in the protein complexes, thereby allowing the appearance of new potentialities in protein. Essentially, HSP90 acts in structural maturation and conformational regulation of various signal-transduction molecules, such as kinases and steroid receptors (46), using, like other chaperones, other regulators and co-chaperones to perform these functions (4).

The sHSP constitute a class of chaperones which have smaller molecular weights and function in an ATP-independent manner. sHSP binds to unfolded/misfolded proteins promoting their folding/refolding in an independent manner or transferring damaged protein to ATP-dependent chaperones, enabling protein refolding or degradation (47). Mutations in sHSP genes can result in protein aggregates formation, namely aggresomes, as observed in desmin-related cardiomyopathy which is triggered by a mutation in sHSP α-β-crystallin (3).

SR is the main organelle where protein folding occurs, being associated with the presence of various chaperones, specifically glucose-regulated protein 78 (GRP78), that belongs to HSP70 family. In fact, dysfunctional SR results in accumulation of misfolded or unfolded proteins. SR chaperones mainly interact with three protein complexes, the activating transcription factor 6 (ATF6), the inositol-requiring enzyme-1 (IRE1) and the protein kinase RNA-like ER kinase (PERK), present in membrane of this organelle. Under normal conditions, GRP78 chaperone associates with these complexes, inhibiting their functions. However, under stress conditions, GRP78 binds to misfolded proteins and dissociates from these complexes, activating them. These protein complexes function as transmembrane sensors that are capable of detecting the accumulation of misfolded or unfolded proteins, and consequently promote an increase expression of chaperones present in this organelle, inhibiting protein synthesis and activating misfolded proteins degradation, in order to restore proteostasis (40, 48).

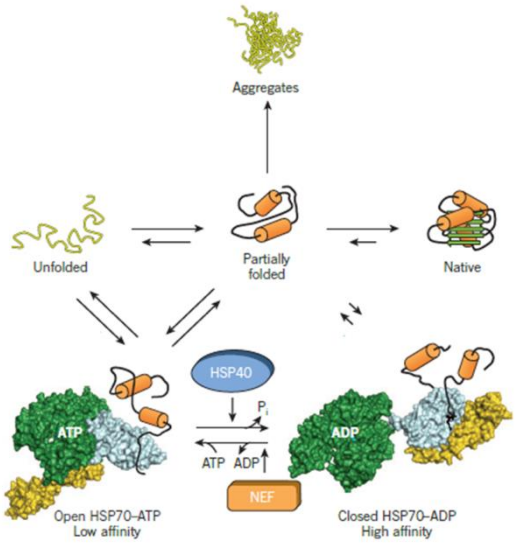

Figure 1: The HSP70 chaperone cycle. General representation of the HSP70 reaction cycle, ATP-dependent, controlled by the HSP40 co-chaperone and nucleotide-exchange factors (NEF).
Adapted from (4).

1.2.2 Ubiquitin-proteasome system

Proteasomal proteolytic pathway is responsible for 90% of intracellular proteins degradation in eukaryotic cells. Therefore, it ensures that not required, misfolded, oxidized and other type of damaged proteins do not accumulate in cells, avoiding their toxic effects. This system exists mainly in two forms, the ATP-ubiquitin independent (20S) and ATP-ubiquitin dependent (26S). The first form is mostly responsible for the degradation of oxidized proteins as resulting from oxidative stress, being this system ATP-ubiquitin independent. On the other hand, ATP-ubiquitin dependent involves protein ubiquitination and its subsequent degradation by 26S

proteasome (49, 50) (Figure 2). Ubiquitination refers to the addition of ubiquitin molecules to a protein substrate through a cascade of enzymatic reactions. This process involves three steps: 1) the ubiquitin-activating enzyme (E1) covalently binds to ubiquitin in an ATP-dependent manner, thereby resulting in its activation; 2) the ubiquitin-conjugating enzyme (E2) transfers ubiquitin molecule from the E1 to itself; 3) the ubiquitin ligase (E3) recognizes the specific protein substrate and transfers ubiquitin molecule from the E2 to a lysine residue present in substrate (Figure 2). The fate of ubiquitinated proteins depends on the length of the ubiquitin chain formed and on the specific sites of lysine residues in ubiquitin used for linkage. When substrates are tagged with four or more ubiquitin molecules (poly-ubiquitination), being attached to each other by their lysine 48, they become targeted for degradation by the 26S proteasome (2, 51).

The 26S proteasome is a multicatalytic complex composed by two 19S regulatory subunits and a 20S proteolytic core. The 19S regulatory subunits, positioned at both ends of the 20S proteolytic core, have as main components chaperones, ATPases and enzymes that remove ubiquitin molecules from substrates (deubiquitinases). Hence, these subunits recognize poly-ubiquitin chains formed in substrates, remove ubiquitin molecules from proteins, promote protein unfolding and its entry into the 20S proteolytic core, in an ATP-dependent manner (41). The 20S core has a cylindrical structure, being constituted by two β-rings and two external α-rings, containing each ring seven unique protein subunits. Three major types of peptidase activities have been identified in 20S proteolytic core: chymotrypsin-like, trypsin-like and caspase-like activities (also designated as peptidylglutamyl-peptide hydrolase), attributed to β5, β2 and β1 subunits, respectively. α-rings avoid the entry of non-specific substrates into the 20S proteolytic core, regulates the exit of peptides products from this core and can directly regulate the assembly and peptidase activities of β-rings (2) (Figure 2). Since UPS is responsible for the majority of protein degradation, loss of its activity may lead to accumulation of dysfunctional proteins and, consequently, cause dysfunction and cellular death.

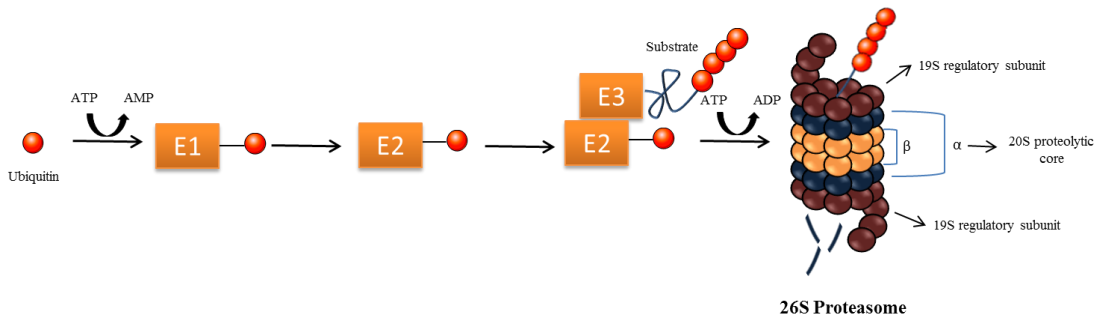

Figure 2: Ubiquitin-proteasome system (UPS). Proteins degradation by 26S proteasome involves the poly-ubiquitination of substrates through a cascade of enzymatic reactions. E1-Ubiquitin-activating enzyme; E2-Ubiquitin-conjugating enzyme; E3-Ubiquitin ligase.

1.2.3 Autophagy

In eukaryotic cells, autophagy is the second protein degradation pathway, although it is the main cellular pathway responsible for degradation of long-lived proteins and unnecessary or dysfunctional organelles (9). This process is essential for degradation of proteins that deposit in larger aggregates, since they cannot be degraded by proteasome proteolytic cavity. Therefore, autophagy is crucial for cellular homeostasis maintenance, protecting cardiomyocytes function (7, 52). There are three distinct forms of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy. Macroautophagy is the most prevalent form of autophagy, so it is usually referred as autophagy. Macroautophagy begins with the formation of a double membrane, the phagophore, around cytoplasmic material and organelles. The fusion of the phagophore expanding ends originates a vacuole, called autophagosome. Then, autophagosome external membrane fuses with lysosome, forming the autolysosome. Subsequently, autolysosome content is degraded by lysosomal enzymes and the remaining components are released to the cytoplasm (53). The main difference between macroautophagy and microautophagy is that, in the latter, cytoplasmic content is directly incorporated into lysosomes through lysosomes membrane invaginations. Chaperone-mediated autophagy consists in the recognition of cytosolic proteins by HSP70, which interact with lysosome-associated membrane protein type 2a (LAMP2A) receptor, present on lysosome membrane, allowing protein translocation into the lysosomes where they will be degraded (54, 55).

Under basal conditions, autophagy activation occurs at low levels, but it can rapidly increase in response to various stimuli, such as starvation, thereby promoting cellular survival through the release of energetic substrates by degradation of cellular constituents (8, 9). Autophagy also seems to function in a selective manner in the elimination of dysfunctional organelles, protein aggregates and intracellular pathogens. Selective types of autophagy must ensure efficient recognition and sequestration of the specific substrates within autophagosomes. This process is mediated by proteins like p62 and neighbor of BRCA1 gene 1 (NBR1), which are capable of recognize ubiquitinated proteins in protein aggregates and connect them to the autophagic membranes (7, 56, 57). Nevertheless, excessive and uncontrolled autophagy activation may promote cellular death, through the destruction of proteins and organelles that are essential to the cells (9). Mitophagy is a particular case of selective autophagy responsible for the degradation of dysfunctional mitochondria. Dysfunctional mitochondria produce less ATP, generate increased amounts of ROS and are more prone to trigger apoptosis, thereby contributing to the development of HF (58, 59). In this context, the elimination of these damaged mitochondria, by mitophagy, is crucial for cardiomyocytes to work properly. Mitophagy is a rigorously controlled and very selective process that includes mitochondrial sequestration by autophagosome, which fuses with lysosome for posterior degradation (59). It has been described that PTEN-induced putative

kinase 1 (PINK1) and Parkin proteins play an important role in mitophagy regulation. Under healthy conditions, PINK1 is imported into mitochondria, through a process dependent of mitochondria membrane potential, being rapidly degraded and maintained at low levels. Upon loss of mitochondrial membrane potential, PINK1 accumulates on the mitochondrial surface, leading to the recruitment of E3 ubiquitin ligase Parkin, that ubiquitinates many mitochondrial outer membrane proteins. The ubiquitinated proteins serve as a signal for p62/SQSTM1 protein, which assists to the recruitment of autophagosome to mitochondria, thereby promoting mitophagy (59, 60).

Autophagy-related proteins (Atg), initially identified and characterized in yeast, are essential for the autophagic process since they are needed for the formation of autophagosome. After autophagy induction, these proteins associate following a hierarchical direction, to first mediate the phagophore formation and then its expansion into autophagosome (57). Autophagy is usually determined by microtubule-associated protein light chain 3 (LC3) protein levels. LC3 protein consists in an autophagy marker which is involved in autophagosome formation. Briefly, LC3 protein is converted to its cytosolic form LC3-I, by Atg4 cysteine protease. Then, LC3-I is activated by Atg7, transferred to Atg3 and conjugated with phosphatidylethanolamine (PE), thereby converting in LC3-II, that is recruited to phagophore double membrane, forming the autophagossome. Therefore, the amount of LC3-II is associated with the number of autophagosomes (53, 55). There are many signaling pathways that play an important role in autophagy regulation, namely mammalian target of rapamycin (mTOR), phosphatidylinositol 3-kinase-I/protein kinase *B* (PI3K-I/Akt), a AMP-activated protein kinase (AMPK) and mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 (MAPK/Erk1/2) (54, 55), as described in Figure 3.

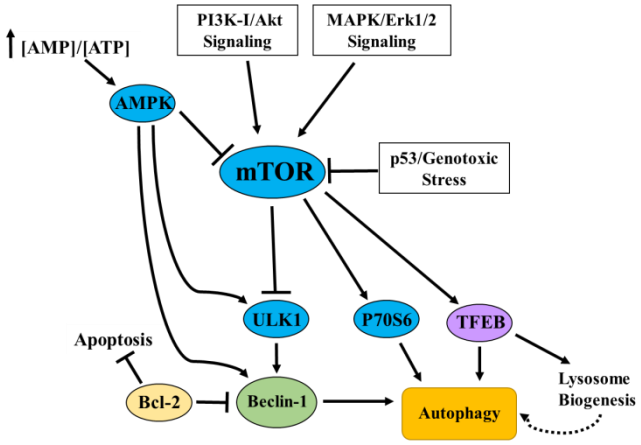

Figure 3: Autophagy regulation. The kinase mTOR is the main regulator of autophagy. mTOR activation promotes autophagy suppression, whereas mTOR inactivation promotes autophagy induction. mTOR can be positively regulated by PI3K-I/Akt and MAPK/Erk1/2 signaling pathways and negatively by AMPK and p53. mTOR regulates autophagy through several pathways, namely acting in ULK1 complex, in kinase p70S6 and in transcription factor TFEB. On the other hand, AMPK is also capable of regulating autophagy by acting directly in ULK1 complex and in beclin-1. Additionally, Bcl-2, an anti-apoptotic regulator, also regulates autophagy by beclin-1 inhibition. AMPK- AMP activated protein kinase; PI3K-I/Akt- phosphatidylinositol 3-kinase/protein kinase B; MAPK/Erk1/2- mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2; mTOR- mammalian target of rapamycin; ULK1- UNC-51 like kinase 1; TFEB- transcription factor EB; P70S6K- Ribosomal Protein S6 Kinase Beta-1; Bcl-2- B-cell lymphoma 2.

In addition to UPS and autophagy, proteases also integrate the main proteolytic systems present in cardiomyocytes. Calpains are cytoplasmic Ca^{2+}-dependent cysteine proteases which participate in several Ca^{2+}-mediated intracellular processes. Although its physiological function has not yet been fully understood, it is known that calpains are involved in cell migration, apoptosis, autophagy and plasma membrane repair. Nevertheless, m-calpains and µ-calpains, expressed in cardiomyocytes, are responsible for degradation of myofibrillar proteins, particularly troponin, tropomyosin, myosin and titin (61). Suppression of calpain-1 activity, by overexpression of calpastatin (a specific endogenous inhibitor for calpain), resulted in development of dilated cardiomyopathy, which is accompanied by a decrease of ubiquitinated proteins, protein aggregates formation, increase in autophagy and destruction of sarcomeres integrity (62). So, calpain-1 activity is essential in maintenance of cardiac function and regulation of specific sarcomeric proteins (62). Additionally, it is known that dystrophins are degraded by calpain. Degradation of dystrophins impairs the integrity of the sarcolemma, leading to cardiac dysfunction and HF. Therefore, calpain inhibition may be a therapeutic target in the regulation of dystrophin degradation, delaying the progress to HF (61).

1.3 Dysregulation of proteostasis in aging

Aging is characterized by progressive decline of cellular proteostasis, and consequently, by accumulation of misfolded and damaged proteins in different organs and tissues (63). In fact, it has been reported the accumulation of protein aggregates in the heart during its natural aging process. Ayyadevara *et al*. reported an aging-dependent increase of detergent-insoluble aggregates isolated from mice hearts (18). Since protein aggregation is suppressed by protein quality control systems, it becomes relevant to clarify how these systems change through aging and its implications on protein aggregates formation, and consequently on cardiac aging (Figure 4). Knowledge of the mechanisms that contribute to cardiovascular aging would have important clinical impact in cardiovascular disease prevention, early diagnosis and development of treatments (18).

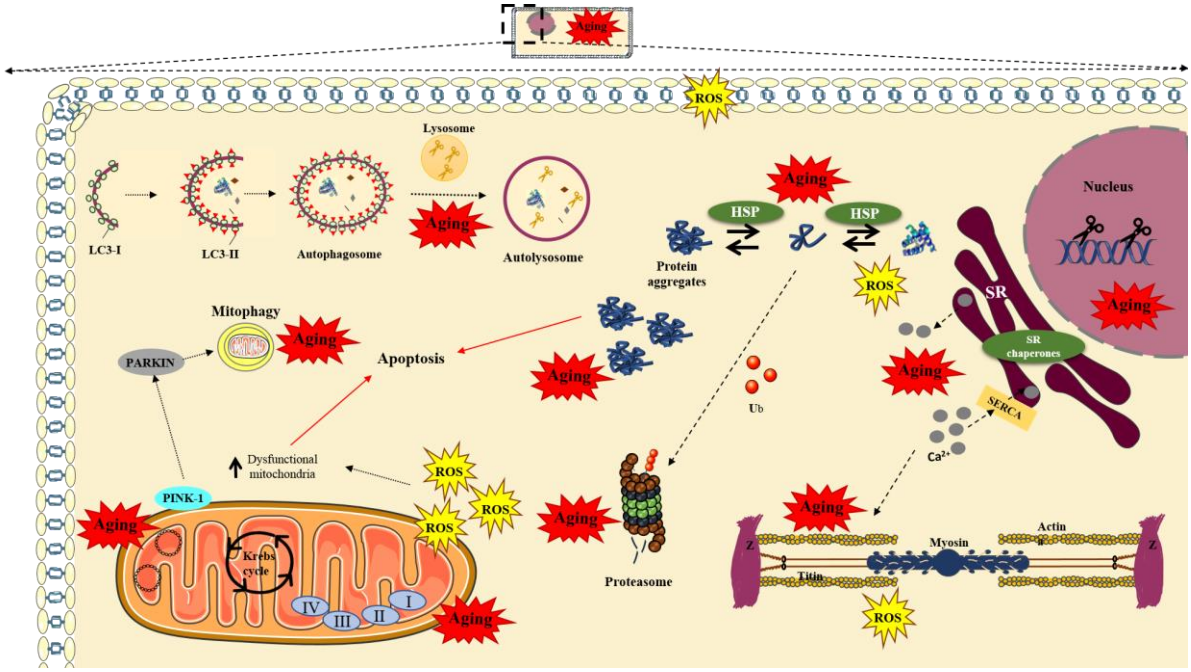

Figure 4: Overview of molecular changes during aging. Aging promotes among other, damage in DNA, RNA, lipids and proteins, changes in Ca^{2+} homeostasis, mitochondrial dysfunction, ROS and oxidative stress increase. Aging is also associated with dysregulation in protein quality control systems, namely, chaperones, UPS, autophagy and mitophagy. All these changes may lead to a disruption in protein homeostasis and, consequently, to the increase of protein aggregates formation.

The induction of HSP synthesis, in response to stress factors, is crucial to maintain the proteostasis balance, preventing the formation of protein aggregates potentially toxic to cardiomyocytes (40). Nevertheless, different studies have shown that the induction of several chaperones in cardiac tissue from aged animals was compromised in response to various stress stimuli, such as temperature increase and physical exercise (64, 65). Thus, aging is associated with a decreased ability for chaperone synthesis, under stress conditions. Furthermore, Ayyadevara and colleagues found that HSP70, HSP90α and HSP90β levels were significantly diminished in aggregates from aged hearts, whereas HSP75, a mitochondrial HSP which protects against cardiac

hypertrophy and fibrosis, was absent (18). At the same time, the authors observed an increase of HSP70 in aggregates from young mice, highlighting its protective functions. Regarding sHSP (HSP-β6, HSP-β7 and HSP-β8), they were more abundant in aggregates from aged hearts. Moreover, tropomyosin levels were increased in aggregates of aged hearts, which can indicate that the rise in the sequestration of tropomyosin within aggregates may contribute to a decrease in cardiac contractility during aging (18).

When protein function cannot be restored from misfolded proteins or aggregates, chaperones direct them to the proteolytic systems (Figure 4). The presence of ubiquitinated proteins in aggregates, a feature of different diseases, can be related to inefficient directing of proteins to proteasomes, resistance to degradation or UPS impairment, thereby contributing to the accumulation of these proteins (63). It has been reported that the activities of ubiquitinating enzymes E1, E2 and E3 did not show any consistent change with age, while the accumulation of ubiquitinated and oxidized proteins is evident both in aging and in diseases associated with aging (66).

Dysfunction of UPS, namely proteasome activity in both hypertrophic cardiomyopathy and failing hearts has been reported, suggesting that proteasome functional insufficiency plays a major role in cardiac pathogenesis (39). The decrease of peptidase activity present in proteasome, namely the chymotrypsin-like activity (67), has been reported in aged tissues, specifically in cardiac tissue. Li *et al.* demonstrated that chymotrypsin-like activity of 20S proteasome was significantly reduced in the cardiac tissue of old rats (25 months) comparative to younger rats (3 months). The decline in 20S proteasome function may be due, in part, to the decrease in the amount of β subunits observed in aged hearts, since these subunits are responsible for the peptidase activities of the proteasome. (68). Furthermore, aging also seems to influence other peptidase activities besides the chymotrypsin-like activity. So, analysis of 20S proteasome purified from rat's hearts with different ages revealed a significantly decrease in peptidylglutamyl-peptide hydrolase and trypsin-like activities on aging, with a consequent reduction of the ability of the 20S proteasome to degrade protein substrates (69). These results suggest that a decrease in proteasome activity occurs during aging.

When protein aggregation is not controlled by chaperone and proteasome activities, autophagy is promoted (5) (Figure 4). The effect of aging in cardiac autophagy has been reported in some studies, although the results revealed to be inconsistent. Taneike *et al.* found in homogenates from C57BI/6 mice hearts with 6, 14 or 26 months that LC3-II levels were lower comparing to the LC3-II levels present in mice with 10 weeks, suggesting that autophagy decreases with age (19). On the other hand, Inuzuka *et al.* observed that LC3-II levels remained constant in both young (3 months) and old (20-24 months) rats, indicating that autophagy does not change during aging (70).

Wohlgemuth *et al.* studied the impact of aging in expression levels of proteins involved in the formation of autophagosome (beclin-1 and LC3-II) and in the degradation by autolysosomes and lysosomes (procathepsin D and LAMP-1). These authors observed an increase of beclin-1 and procathepsin D expression and a decrease of LAMP-1 expression in hearts from old rats (26 months) comparing with young controls (6 months). In addition, they also verified that LC3-II protein expression was significantly increased in older rats (71). The differences in results between the studies could be due to experimental variables, such as animal model features. Besides, it is important to refer that these studies were based on determination of LC3-II levels, however, the increase in LC3-II does not always represent an induction of autophagy, but it can indicate either an increase in autophagosome formation or a blockade of autophagy pathway. In order to evaluate autophagy induction it is essential the determination of autophagic flux, since it is representative of the whole process, namely from the inclusion of substrate within the autophagosome, its delivery to lysosomes (through fusion of autophagosome with lysosome) and the release of the resulting degradation macromolecules into the cytoplasm (53). Therefore, more studies are needed to characterize autophagic flux changes in cardiomyocytes through aging.

As mentioned before, aging is associated with the increase of dysfunctional mitochondria. Thus, the elimination of damaged mitochondria by mitophagy is important during aging. It has been described that the process of Parkin-mediated mitophagy is impaired in aged hearts (60). In fact, the deletion of Parkin promotes the accumulation of dysfunctional mitochondria in mice hearts with aging (72). Moreover, overexpression of this protein leads to an increase of mitophagy in aged cardiomyocytes, accompanied by a decrease of dysfunctional mitochondria and an improvement in cardiac function associated with aging. Therefore, the activation of mitophagy could be a novel target to attenuate the development of cardiovascular diseases induced by aging (60).

Many studies also shown that changes in lifestyle can efficiently retard aging through improvement of autophagy. For instance, He *et al.* demonstrated that physical exercise increases heart autophagy (73) and has beneficial effects in prevention of both cardiovascular diseases and age-related diseases (74). Furthermore, caloric restriction is also a potent inducer of autophagy, with cardioprotective effects (71). Although physical exercise and caloric restriction manifest beneficial effects in aging prevention, pharmacological induction of autophagy is often used to prevent aging and several age-related diseases (54). Resveratrol has been investigated as a potential therapeutic strategy, although its beneficial effects in cardiomyocytes seem to be dose dependent. Therefore, it is essential to develop pharmacodynamics studies to better understand their cardioprotective or cardiotoxic effects (75). Rapamycin, an mTOR inhibitor, is also considered promising in aging treatment since it can provide functional benefits in aged hearts (76, 77). Wilkinson *et al.* have shown that rapamycin-treated rats presented a delay in the development of

many diseases associated to aging, including degenerative changes in myocardium. On the other hand, chronic treatment with this drug caused harmful side-effects that can impair functions in some tissues (77). In conclusion, there is still a long way to go to fully understand the role of protein aggregation in proteostasis deregulation induced by aging.

1.4 Dysregulation of proteostasis caused by doxorubicin

The pathophysiological mechanisms associated with Doxo-induced cardiotoxicity have not been fully elucidated. Nevertheless, previous studies have consistently reported the cumulative-dose related cardiotoxic profile of Doxo, a feature that limits the extent to which it can be used safely. Moreover, its cardiotoxicity is also dependent of the administration schedule, the concomitant use of other cardiotoxic therapies, history of cardiac diseases, age and gender (13, 78). Anthracycline-induced cardiotoxicity presents both acute and chronic effects. Acute cardiotoxicity occurs during or immediately after Doxo treatment, being rare and not dose dependent (79). Doxo-induced cardiotoxicity may become clinically evident later in the course of chemotherapy and within one year after therapy finalization (early-onset chronic cardiotoxicity), or more than one year after its completion (late-onset chronic cardiotoxicity) (13). In the latter, cardiac abnormalities are usually persistent and progressive, eventually triggering cardiac cells death (23, 78). This injury is typically characterized by the development of left ventricular systolic dysfunction, which subsequently progresses towards dilated cardiomyopathy and HF (23, 80).

Over the last few years, it has been demonstrated that Doxo is able to induce changes in chaperones, UPS as well as in autophagy processes (Figure 5). The dysregulation of protein quality control can promote an increase in protein aggregates formation, which may induce toxic effects in cardiomyocytes. As mentioned above, evidences suggest a link between anthracycline-induced cardiotoxicity and changes in chaperone expression (22). For instance, cardiac biopsies from rabbits treated with other anthracycline, daunorubicin (3mg/kg, weekly, 10 weeks), showed an increase in the expression of various chaperones and proteins involved in chaperone-mediated autophagy, as well as an activation of proteolytic machinery (81). Moreover, Doxo-induced cardiotoxicity was also related to SR chaperones dysregulation/inhibition, such as GRP78 chaperone. In fact, Fu *et al.* showed that Doxo induced an apoptotic response in cardiomyocytes, through the activation of ER mechanotransmembrane sensors, accompanied by the inhibition of GRP78 expression, which in turn potentiates the increase of ER stress (48).

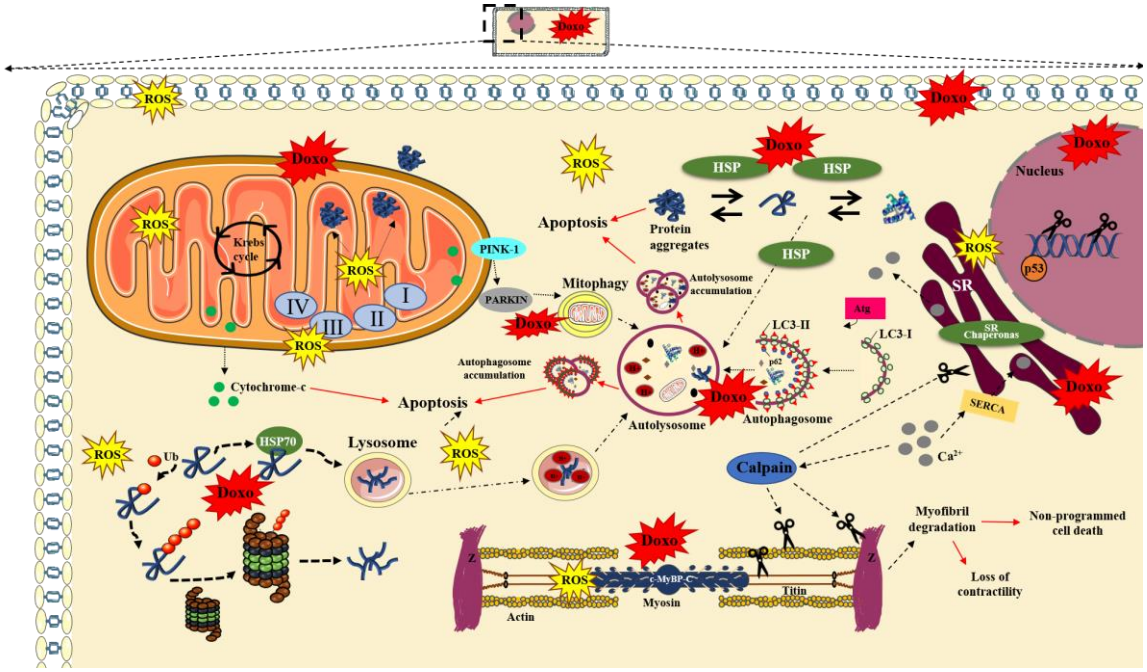

Figure 5: Overview of molecular mechanisms in Doxo-induced cardiotoxicity. Doxo promotes among other, DNA damage, dysregulation of Ca^{2+} handling, myofibrillar degradation, mitochondrial dysfunction, ROS and oxidative stress increase. This drug is also capable to dysregulate protein quality control systems, namely, chaperones, UPS, autophagy, mitophagy and calpain. All these changes may lead to a disruption in protein homeostasis and, consequently, to the protein aggregates formation.

For this reason, recently, chaperones have been intensively studied as potentially new therapeutic targets for cardiac diseases induced by antineoplastic agents. Based on experiments performed in Doxo-induced cardiotoxicity animal model, in which a decrease of HSP20 expression in cardiac tissue was observed, Fan *et al.* demonstrated that overexpressing this chaperone triggered an improvement in Doxo-induced deleterious effects, including higher resistance to apoptosis, enhanced cardiac function and increase animal survival after chronic Doxo treatment (82). These authors also showed that the mechanisms underlying these beneficial effects are associated with the preservation of Akt phosphorylation/activity, as well as the decrease in oxidative stress induced by Doxo (82, 83).

The specific effects of Doxo in UPS have been already reported, although some results remain controversial. Briefly, when Doxo is administrated it diffuses through cellular membrane into the cytosol where it interacts with the proteasome. The complex formed is translocated to the nucleus, and there Doxo is released from proteasome which allows its binding to DNA (Figure 5) (84). Protein ubiquitination is generally a requirement for proteins to be degraded by the 26S proteasome, being that the specificity of ubiquitination resides mainly in ubiquitin ligase E3, as already mentioned (50, 51). In H9C2 cardiomyoblasts cell culture and in hearts from tumour-bearing GFP-LC3 mouse model, the treatment with Doxo induced a significant increase of E3

ligases RING-finger protein-1 (MURF-1) and muscle atrophy F-box (MAFbx) expression, which tag by ubiquitination, myofibril proteins in skeletal and cardiac muscle. Moreover, protein ubiquitination was also significantly augmented both *in vitro* and *in vivo* models, with proteins tagged with poly-ubiquitin chains displaying the higher range of molecular weights and possibly indicating degradation of proteins involved in cardiac muscle structure, by the 26S proteasome (85). However, Doxo caused a significantly reduction on proteasome chymotrypsin-like activity, indicating that this drug is able to inhibit the proteasome (85). The mechanism by which Doxo alters proteasome activity appears to be regulated by the amount of Doxo molecules binding onto the proteasome (86), namely, proteasome activation at lower concentrations and its inhibition at higher Doxo concentrations (25, 87).

Doxo is also capable of inducing post-translational modifications, such as carbonylation, in some sarcomeric proteins (Figure 5), thereby increasing the probability of these proteins to be degraded by proteasome. Recently, it was demonstrated in hypertensive rats that Doxo-induced oxidative stress induces high levels of carbonylation in sarcomeric proteins, more evidenced in myosin binding protein C (MyBPC). In addition, an increase in MyBPC degradation by proteasome was observed, resulting in a reduction of MyBPC levels in cardiomyocytes, as well as in impairment of the interaction of this protein with actin, thereby affecting cardiac cell contractility (88).

With regard to Doxo effects on cardiac autophagy they are still controversial. For instance, a study demonstrated that Doxo promotes autophagic flux in cardiomyocytes. In fact, treatment with bafylomicin-A, a lysosomal H-ATPase inhibitor that impairs the fusion of autophagosome with lysosome, originated a significant increase in LC3-II levels in cardiomyocytes treated with Doxo compared with their controls. In addition, the increase in autophagy induced by Doxo potentiated cardiomyocytes death, suggesting that autophagy activation contributes to Doxo-induced cardiotoxicity (89). In contrast, several studies appear to contradict these results by reporting that Doxo inhibits autophagic flux in cardiomyocytes rather than induces it (90, 91). This result can be explained by the effect of Doxo on the Akt/mTOR pathway activation which leads to suppression of autophagy. The significantly decrease in proteins involved in this process like beclin-1, LC3 and p62, after Doxo treatment, also corroborate this finding (91). Further, the block of autophagic flux in cardiomyocytes can be a consequence of Doxo's capacity to inhibit lysosomal acidification, and consequently, lysosome function. In fact, neonatal rat cardiac myocytes treated with Doxo demonstrated an increase in lysosome pH from 4.6 to 5.2 thereby compromising lysosome hydrolases activities and proteolysis process. So, blocking the autophagic flux leads to autolysosome accumulation, promoting an increase of ROS, which contribute to Doxo-induced cardiotoxicity (90). Interestingly, the same authors showed that inhibition of the autophagic flux

induced by Doxo seems to be transitional (90). Also, it was demonstrated that Doxo is capable of impairing cardiac autophagy by suppressing TFEB expression, which regulates the transcription of genes related to autophagy and lysosomal biogenesis and function. In fact, the loss of TFEB in cardiomyocytes led to a reduction in macroautophagy protein expression, inhibition of autophagic flux, impairment in lysosomal cathepsin B activity and activation of cell death. Whereas TFEB overexpression in Doxo-treated cardiomyocytes augments lysosomal proteolysis, decreases ROS overload, attenuates cleaved caspase-3 expression and improves cardiomyocyte viability (92).

Efficient and functional mitochondria networks are essential to myocardial contraction as well as for the cardiomyocytes survival. Doxo triggers a cellular cascade leading to mitochondria damage, that eventually culminates with cell death (Figure 5). Since mitochondrial density is greater than 35% in cardiomyocytes, Doxo-induced mitochondrial dysfunction may cause a severe lack of energy supply to these cells (93). A recent study, using *in vitro* and *in vivo* models, indicates that exposure to Doxo promotes a decrease in mitophagy mediated by Parkin protein. This reduction seems to be due to the interaction between cytosolic p53 protein and Parkin protein, which inhibits the translocation of the latter to the dysfunctional mitochondria. Thus, elimination of dysfunctional mitochondria by mitophagy seems to be compromised in cardiotoxicity, leading to cardiac dysfunction (60). However, in H9C2 cardiac myoblasts treated with Doxo, Weiner *et al.* reported an increase in mitochondrial fragmentation and in mitophagy. These results were accompanied by an increase of protein levels and mitochondrial translocation of Parkin and PINK1 2 hours after Doxo treatment, however only PINK1 protein levels maintained higher during 24 hours. Considering this, the same authors studied the effect of Parkin overexpresssion (via adenovirus), that accelerated Doxo-induced mitochondrial morphology changes and induced mitophagy, which in turn was associated with an increase in cell death. On the other hand, Parkin genes inhibition (via siRNA) prevented Doxo-induced mitophagy and diminished apoptosis markers levels (94).

Regarding these evidences, Doxo has the ability to induce modifications in protein quality control systems, being associated with the formation of protein aggregates, which may contribute to the cardiotoxicity induced by this anthracycline. Since the existing information is still controversial, it is essential to carry out further studies in order to understand the role of Doxo in protein aggregates formation and its relationship with the cardiotoxicity.

1.5 Methodological approaches to study protein aggregates

The characterization of protein aggregates can provide valuable insights into the understanding of cardiomyocytes' remodeling in response to aging and mechanisms underlying cardiotoxicity. Several methodological approaches have been proposed. Bastos *et al.* (5) highlighted the potential of mass spectrometry (MS) for protein aggregation research once it allows protein identification and characterization from complex mixtures of biological origin. Protein aggregates are not homogeneous in regard to their size, composition and morphology. Such heterogeneity makes the study of protein aggregation challenging (95). The identification of aggregates components is troubled by difficulties in the isolation of protein aggregates, thereby no standard method was yet proposed. The generally approach for the isolation of proteins aggregates are based on the fact that insoluble aggregates, namely amyloids, are resistant to strong detergents. This property allows the isolation of amyloids by centrifugation in the presence of detergent (96). Different detergents have been proposed to isolate protein aggregates, such as sodium dodecyl sulphate (SDS) or sodium N-lauroylsarcosinate (sarcosyl) (18, 97). Some studies demonstrated that amyloids can be isolated from yeast cell lysates by sedimentation in the presence of SDS, showing the role of SDS in the isolation of protein aggregates (96). However, it has been reported that the use of a milder detergent, like sarcosyl, instead SDS that presents a strong denaturant effect and impairs protein's native conformation, allowed the purification of amyloid aggregates which cannot withstand SDS treatment (97). Furthermore, in cardiac tissue, the use of sarcosyl, was also shown to be efficient in the isolation of protein aggregates-enriched fractions (18). Resolubilization of insoluble aggregates is a crucial step to ensure adequate separation of proteins in SDS polyacrylamide gel electrophoresis (SDS-PAGE). Due the high insolubility of protein aggregates, the use of high percentage of strong detergents to protein aggregates solubilization is essential to achieve maximal protein recovery (98). Gel electrophoresis is commonly used for estimating protein size, identifying proteins and determining sample purity (95). SDS-PAGE is a simple and very frequently used approach to separate and analyze the extracted protein aggregates (95). Also, this separation method allows downstream identification of proteins by MS after in-gel digestion. This method is preferable to the alternative in-solution digestion, because many MS interferents are trapped inside the gel, while peptides can be easily recovered from the polyacrylamide matrix (99, 100). The study of protein aggregates is still a major challenge, taking into account that protein aggregation is a dynamic process that can be influenced by several factors, such as temperature, protein concentration and pH (95). Therefore, development/optimization of methods for isolation of the aggregates and identification of the associated proteins can be essential for understanding the cellular processes underlying the aggregation-driven toxicity.

2. AIMS

The main objectives of the work carried out were to investigate the potential involvement of protein aggregation in the pathophysiological mechanisms underlying the cardiovascular changes associated with aging, as well as to understand the mechanisms by which the protein aggregation promotes Doxo-induced cardiotoxicity. For this purpose, the first goal was to optimize the methodology for protein aggregates enrichment isolated from left ventricle (LV) of an animal model of aging and an animal model of cardiotoxicity. Then, the second goal was to identify proteins from aggregates-enriched fractions by SDS-PAGE followed by liquid chromatography-tandem mass spectrometry (GeLC-MS/MS). The last goal was to understand if the biological processes associated to the proteins present in aggregates differ between young and aged rats LV and between LV from healthy and Doxo-treated animals, as well as their biological implications.

3. MATERIALS AND METHODS

3.1 Animal model

3.1.1 Aging animal model: Left ventricular samples from a rat model of aging previously established in our laboratory were used for studying protein aggregation. Briefly, young and aged male Wistar Kyoto rats (WKY, n=5 each group) (Charles River, US) were maintained for 25 weeks-old and 20 months-old, respectively, in individually ventilated chambers, in a controlled environment with a 12:12h light/dark cycle at 20 ± 2ºC room temperature. Water and standard diet were provided *ad libitum*.

3.1.2 Doxo-induced cardiotoxicity model: Left ventricular samples from a rabbit model of cardiotoxicity previously established in our laboratory were used for studying protein aggregation. Briefly, a well-documented regimen was used for the induction of HF due to doxorubicin toxicity (101). Male New Zealand white rabbits (n=5) received a bolus of doxorubicin (Doxo group, 1mg/Kg, iv) via marginal ear vein, twice a week for 8 weeks, followed by a washout period of 1 week. Control rabbits (n=5) received 0.9% saline (vehicle) in equivolumetric doses over the same period. The animals were housed in stainless steel cages in a controlled environment with a 12:12h light/dark cycle at 20 ± 2ºC. Water and standard diet were provided *ad libitum*.

At the end of the experiments, rats and rabbits were euthanized under anesthesia and subsequently LV were collected, snap frozen in liquid nitrogen and stored at -80ºC for protein aggregates studies. Experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication no. 85–23, revised 2011) and was approved by the ethics committee of the Faculty of Medicine of University of Porto.

3.2 Baseline protocol for protein aggregates extraction and purification

The methodological approach for the isolation of protein aggregates-enriched fractions was based on the Ayyadevara *et al.* protocol (18). In order to optimize the technique, different conditions were tested: 1) tissue homogenization method (potter or beads); 2) DNase treatment; 3) protein precipitation method (TCA/acetone or acetone); 4) tissue amount; 5) sample type (failing and healthy myocardium); 6) different animal species (rat and rabbit). The optimized protocol is described in “Results” section.

3.3 In-gel protein digestion

For protein identification by LC-MS/MS, several steps were performed. First, lanes of the 12% SDS-PAGE gel were cut and sliced into 16 sections and each section was washed two times with 50 mM ammonium bicarbonate (NH_4HCO_3) for 30 minutes and acetonitrile (ACN) for 30 minutes each, to remove the staining and contaminants. Gel sections were dehydrated for 1 hour with ACN and dried in a SpeedVac (2000 rpm, 10 minutes, 4ºC). The proteins present in each section were reduced with 10 mM dithiothreitol (DTT) for 30 minutes at 60ºC, alkylated using 55 mM iodoacetamide (IAA) for 30 minutes at room temperature and enzymatically digested with trypsin (Pierce Trypsin Protease, MS Grade, Thermo Scientific™), diluted in 50 mM ammonium bicarbonate, for 16 hours at 37ºC. The digestion was stopped with 10% formic acid and peptides were extracted from the gel fraction using 10% formic acid:ACN (1:1) for 30 minutes. Then, peptides were washed in ACN for 30 minutes and dried using vacuum. Finally, the peptides were desalted and cleaned up using OMIX Tip C18 (Agilent) (following the manufacturer's instructions), dried and stored at -80ºC before LC-MS/MS analysis.

3.4 Protein identification by GeLC-MS/MS

The peptide mixes were analysed using an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific, San Jose, CA, USA) coupled to an EasyLC (Thermo Scientific (Proxeon), Odense, Denmark). Peptides were loaded directly onto the analytical column and were separated by reversed-phase chromatography using a 50-cm column with an inner diameter of 75 μm, packed with 2 μm C18 particles spectrometer (Thermo Scientific, San Jose, CA, USA). Chromatographic gradients started at 95% buffer A and 5% buffer B with a flow rate of 300 nl/min, gradually increased to 22% buffer B in 79 min and then to 35% buffer B in 11 min. After each analysis, the column was washed for 10 minutes with 5% buffer A and 95% buffer B. Buffer A: 0.1% formic acid in water. Buffer B: 0.1% formic acid in acetonitrile.

The mass spectrometer was operated in DDA mode and full MS scans with 1 micro scans at resolution of 120.000 were used over a mass range of m/z 350-1500 with detection in the Orbitrap. Auto gain control (AGC) was set to 2E5 and dynamic exclusion to 60 seconds. In each cycle of DDA analysis, following each survey scan Top Speed ions with charged 2 to 7 above a threshold ion count of 1e4 were selected for fragmentation at normalized collision energy of 28%. Fragment ion spectra produced via high-energy collision dissociation (HCD) were acquired in the Ion Trap, AGC was set to 3e4, isolation window of 1.6 m/z and maximum injection time of 40 ms was used. All data were acquired with Xcalibur software v3.0.63.

MS/MS data from rat and rabbit samples were searched against SwissProt rat database (April, 2017) and rabbit database (June, 2017), respectively using the search algorithm Mascot version 2.5.1 (Matrix Science, http://www.matrixscience.com/). Only peptides showing a false discovery rate (FDR) lower than 5% were retained. Peptide intensities were normalized as a function of the median and positive identification required a minimum of two peptide matches. The LC-MS/MS analysis was performed by the CRG/UPF Proteomics Unit of the Center for Genomic Regulation (CGR), University Pompeu Fabra (UPF).

3.5 Bioinformatic and Statistical analysis

ClueGO, a Cytoscape plug-in, was used to create molecular networks, which allowed the visualization of the biological processes associated to the identified proteins, and therefore help with attributing a biological meaning to the aggregated proteins. The proteins of different groups were uploaded in ClueGO as two separate clusters. This procedure was done for both animal models. Bioinformatic tool, DisGeNET was used to visualize networks showing the associations of proteins to human diseases. This analysis, that integrates data from expert curated repositories, was performed for proteins exclusively identified in Aged and Doxo groups. To calculate the grand average of hydropathy (GRAVY) value for protein sequences, online program GRAVY calculator (http://www.gravy-calculator.de/) was used. Online program Jvenn (http://bioinfo.genotoul.fr/jvenn/) was also used to create Venn diagrams. Optical density was calculated using Image Lab program (Supplement A and B).

For differential protein analysis, we first selected only proteins identified in all animals (n=5 in both Young and Aged group; n=5 in both Control and Doxo group). Then, a volcano plot was done, with each point representing a protein, and the x-axis being the $\log_2$ (fold-change) and y-axis being $-\log_{10}$ (*p*-value*)* from a t-test. Each sample protein profile was normalized by dividing each protein area by the median of that profile. Then, the fold-change of proteins was computed by taken the ratio of the Aged samples and Young samples. The same procedure was applied to proteins from Control and Doxo groups. To improve statistical analysis insight, the Cohen's d or magnitude assessment, calculated as the difference between the means of two groups divided by pooled standard deviation of the groups as well as its 95% confidence interval (CI) were performed (Supplement C and D). Proteins whose CI do not include zero were selected. Proteins were considered markedly increased or decreased if they have $-\log_{10}$ *p*-value>1.301 ($\log_{10}$ of 0.05), $\log_2$ (fold-change) <-1 or >+1 and 95% magnitude CI different from zero. *t*-test was used to evaluate the differences between groups, making use of GraphPad Prism software (version 6.0). Results are presented as mean ± Standard Error of Mean (SEM). $p<0.05$ was considered significant.

4. RESULTS

4.1 Methodological optimization for the isolation and characterization of the protein aggregates-enriched fractions

As mentioned before, the first goal of this thesis consisted in optimizing the technique for protein aggregates enrichment, based on the methodological approach described in the study of Ayyadevara *et al*. (18). We present below the most relevant experiments carried out, which allowed the validation of the final protocol:

a) Protein precipitation method (TCA/acetone)

In the first test, heart tissue samples were homogenized mechanically in RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2.5% sodium deoxycholic, 1% NP40, 1 mM EDTA and protease inhibitor cocktail) using the potter homogenizer. Afterward, the lysates were incubated with DNase for 15 minutes at 37ºC and protein concentration was determined using DC Protein Assay Kit. Protein precipitation was performed in one-half of the sample, using TCA/acetone method. Briefly, TCA and ice-cold acetone was added to the sample in a ratio of 1:8:1, and proteins were precipitated for 1 hour at -20ºC. After centrifugation (18,000 x *g*, 15 minutes, 4ºC), pellet was washed with ice-cold acetone. Then the pellet was dried, suspended in HEPES buffer (0.1 M HEPES, pH 7.4, 1% sarcosyl, 5 mM EDTA and protease inhibitor cocktail) and centrifuged to obtain detergent-insoluble fraction. Finally, the pellet was dissolved in 10% SDS and the proteins were separated by SDS-PAGE. The results obtained are shown in Figure 6.

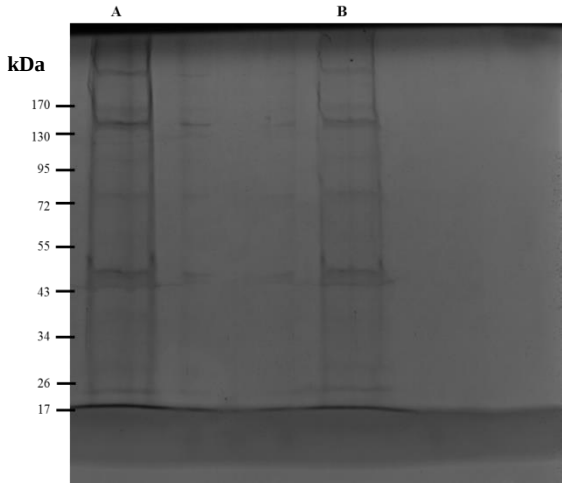

Figure 6: Optimization of the precipitation method with TCA/acetone. SDS-PAGE of the protein aggregates-enriched fractions obtained from healthy myocardial sample. Proteins present in half of the tissue (15 mg) were not subjected to TCA/acetone treatment (A), while those present in the other half (15 mg) were treated with TCA/acetone (B).

Based on SDS-PAGE profiles, proteins not subjected to TCA/acetone treatment (Figure 6A) had, apparently, greater band intensities, indicating higher protein amount, comparing to proteins treated with TCA/acetone (Figure 6B). Therefore, this protein precipitation method did not show apparent advantage. Moreover, the poor intensity of the bands may indicate that a higher amount of tissue is necessary.

b) Sample type and quantity

A second test was designed to evaluate different sample types, i.e., healthy and failing myocardial tissue (Figure 7). In this experiment, TCA/acetone protein precipitation method was excluded.

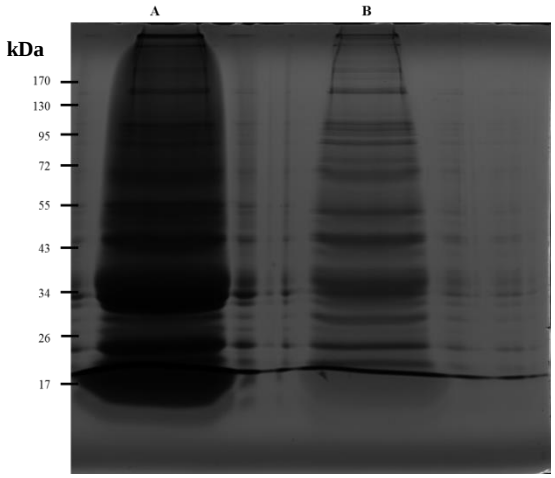

Figure 7: Optimization of the protein extraction protocol for different types of samples (healthy and failing myocardium). SDS-PAGE of the protein aggregates-enriched fractions obtained from a failing (120 mg) (A) and a healthy rat heart (280 mg) (B).

Figure 7, shows protein aggregates-enriched fractions in both failing and healthy myocardium.

c) Sample quantity and protein precipitation method (acetone)

To improve the profile and purity of the sample, another method for protein precipitation using ice-cold acetone overnight at -20ºC, followed by centrifugation at 14,000 x *g* for 15 minutes at 4ºC was tested. Additionally, in the aging animal model, one of the major limitations was the amount of sample available. Hence, the next experiment had two aims: evaluate a softer protein precipitation method using ice-cold acetone and smaller amounts of tissue sample (20, 100, 200 and 400 mg) (Figure 8).

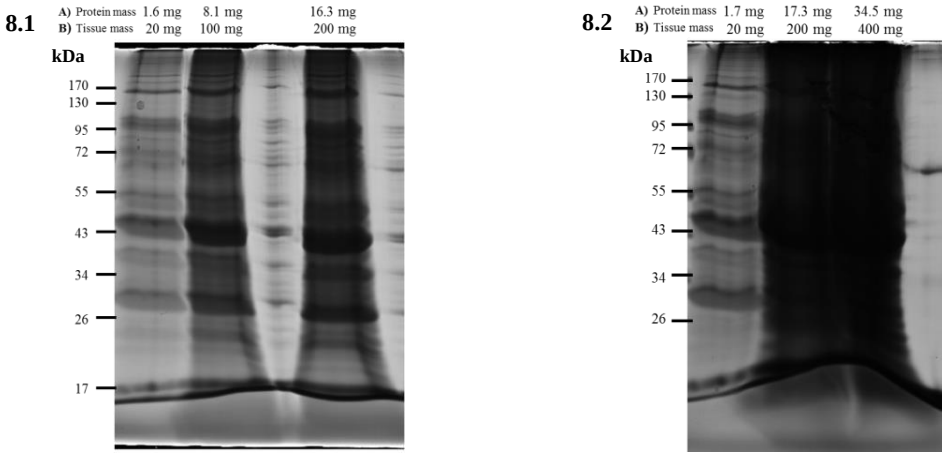

Figure 8: Optimization of the smallest quantity of tissue. SDS-PAGE of the protein aggregates-enriched fractions obtained from cardiac tissue of failing (8.1) and healthy rat heart (8.2). Corresponding protein mass (A) to the different amounts of tissues tested (B).

As presented in Figure 8, all quantities of tissue tested seemed to yield sufficient amount of protein aggregates-enriched fractions, as seen by intensity of the bands. Nevertheless, when starting with at least 100 mg of tissue, a consistent dragging across the lane (Figure 8) was observed, evidencing protein overload. In both tests, with the smallest amount of tissue tested (20 mg) and the ice-cold acetone precipitation method, a "clean" profile of aggregated proteins was obtained.

In order to evaluate the possibility of working with even smaller amounts of sample, 5, 10, 20 and 30 mg of tissue were tested. Results obtained are shown in Figure 9.

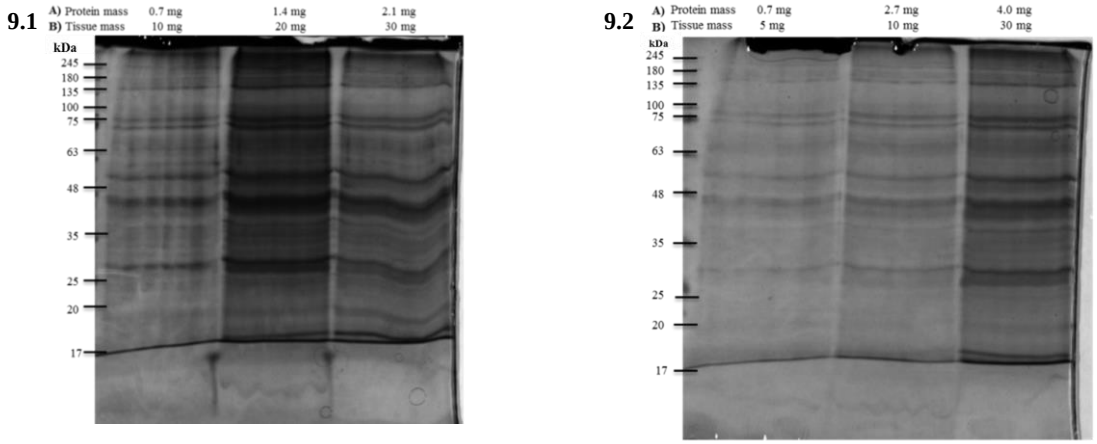

Figure 9: **Optimization of the smallest quantity of tissue.** SDS-PAGE of the protein aggregates-enriched fractions obtained from cardiac tissue of failing (9.1) and healthy rat heart (9.2). Corresponding protein mass (A) to the different amounts of tissues tested (B).

Both experiments showed enriched fractions in aggregated proteins independently of the amount of protein tested.

d) Potter *vs* beads based mechanical homogenization

In attempt to maximize protein extraction from the myocardial sample, a new type of tissue homogenization was used instead of the potter-based mechanical homogenization system. This time, a bead system was used instead of the potter homogenizer (Figure 10).

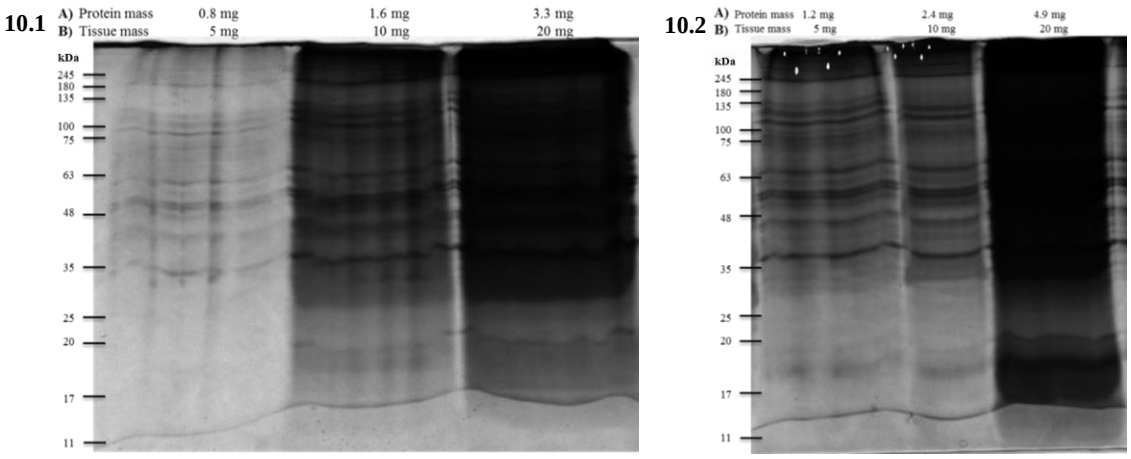

Figure 10: Optimization of the homogenizing method. SDS-PAGE of the protein aggregates-enriched fractions obtained from cardiac tissue of failing (10.1) and healthy rat heart (10.2). Corresponding protein mass (A) to the different amounts of tissues tested (B).

With beads homogenizing method, it was possible to obtain a "cleaner" profile of aggregated proteins using only 5 and 10 mg of tissue (Figure 10). Besides, using the same type and the same amount of tissue, the beads system increased the protein extraction yield in comparison to the potter homogenizer (Table 1). Failing myocardium showed more than a 2-fold increase in protein extraction yield, while in the healthy myocardium the increase was over 1.5-fold (Table 1).

Table 1: Protein extraction yield obtained from different amounts and types of sample using distinct tissue homogenization methods. Corresponding protein yield (%) (total protein mass (mg) / tissue mass (mg) x 100) to the different amounts of tissues tested (mg) obtained using two types of tissue homogenization (potter (P) or beads (B) system). Tests were performed using cardiac tissue from failing and healthy rat hearts.

	Tissue mass (mg)	***Protein mass (mg)***	***Protein Yield (%)***
Failing myocardial sample	10	0.7 (P)	7
		1.6 (B)	16
	20	1.4 (P)	7
		3.3 (B)	16.5
Healthy myocardial sample	5	0.7 (P)	14
		1.2 (B)	24
	10	1.3 (P)	13
		2.4 (B)	24

e) Optimization of aggregate proteins amount for GeLC-MS/MS analysis

After optimizing the conditions for protein aggregates enrichment, the amount of existing protein in these aggregates was evaluated. Therefore, in the next experiment, the method's reproducibility was tested and the amount of aggregated proteins was assessed to determine its suitability for GeLC-MS/MS analysis. Hence, two different tissue amounts (5 and 10 mg) were tested (Figure 11). The impact of acetone-based precipitation method on the aggregated proteins mass was also evaluated (Table 2).

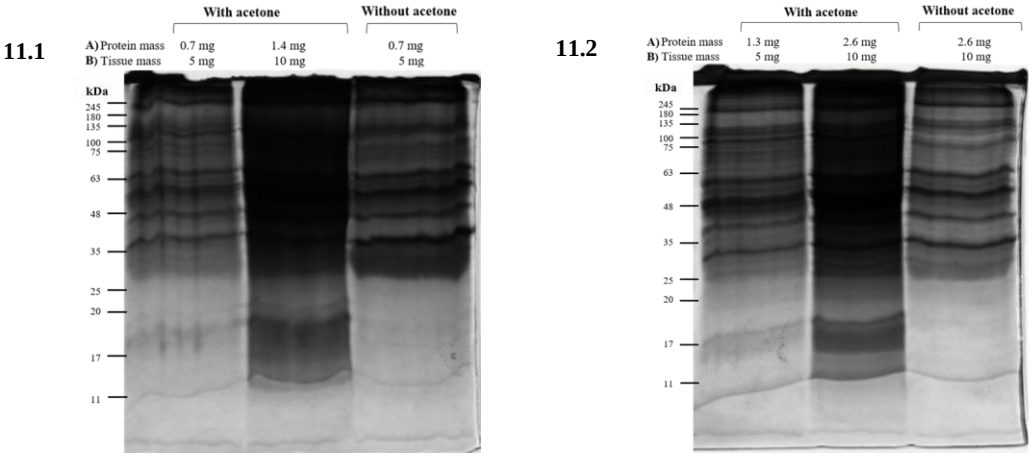

Figure 11: Evaluation of protein precipitation method and optimization of smallest amount of tissue for an adequate protein aggregates enrichment. SDS-PAGE of the protein aggregates-enriched fractions obtained from cardiac tissue of failing (11.1) and healthy rat heart (11.2), previously treated or non-treated with acetone. Corresponding protein mass (A) to the different amounts of tissues tested (B).

Table 2: Protein extraction yield obtained from different amounts of samples. Corresponding protein yield (%) (protein aggregates-enriched fraction mass (mg) / total protein mass (mg) x 100) to the different amounts of tissues tested (mg), obtained with or without acetone treatment for protein precipitation. Tests were performed using cardiac tissue from failing and healthy rat hearts.

		With acetone		***Without acetone***
Failing myocardial sample	Tissue mass (mg)	5	10	5
	Protein aggregates-enriched fraction mass (mg)	0.493	1.246	0.420
	Protein yield (%)	70	88	59
Healthy myocardial sample	Tissue mass (mg)	5	10	10
	Protein aggregates-enriched fraction mass (mg)	0.482	1.288	0.870
	Protein yield (%)	37	49	33

As shown in Figure 11, the method was reproducible as the band profile obtained was similar to previous gels, as seen, for example, by the presence of the bands with estimated molecular weights of 245, 100 and 75 kDa. Moreover, 5 mg of tissue is sufficient to proceed to GeLC-MS/MS. Besides, treatment with acetone increased the protein extraction yield in both

failing and healthy myocardial samples when compared to those untreated, thereby showing the advantage of using a softer precipitation method (Table 2).

f) Protocol reproducibility in myocardial samples from New Zealand rabbits

The aim of this thesis was to study protein aggregates-enriched fractions from two different animal models, which imply distinct species. All protocols, mentioned before, were tested in myocardial samples from rats (*Rattus norvegicus*), confirming its suitability. Thus, we needed to validate the optimized protocol in myocardial samples from New Zealand rabbits (*Oryctolagus cuniculus*) (Figure 12).

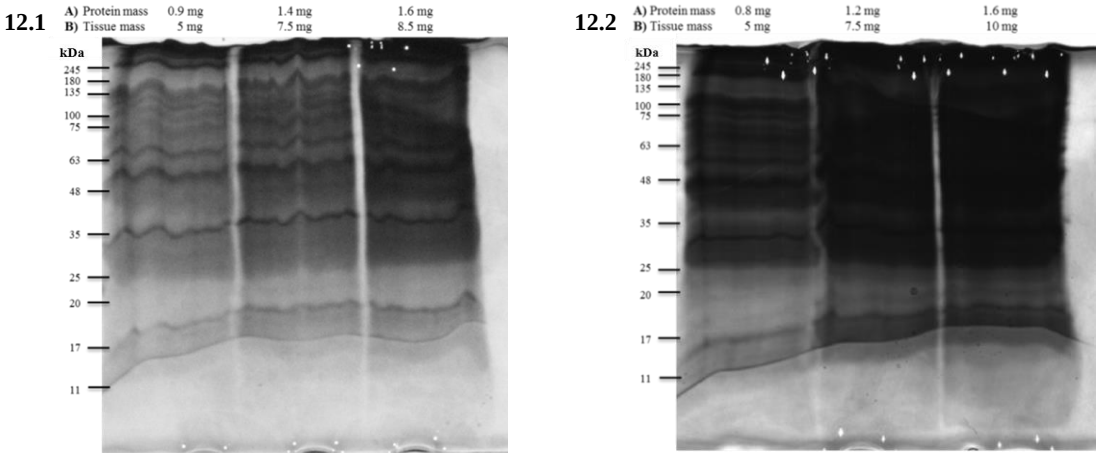

Figure 12: Suitability of the optimized protocol for myocardial samples from New Zealand white rabbits. SDS-PAGE of the protein aggregates-enriched fractions obtained from New Zealand white rabbits cardiac tissue treated with vehicle (12.1) and treated with Doxo (12.2). Corresponding protein mass (A) to the different amounts of tissues tested (B).

Table 3: Corresponding protein aggregates-enriched fraction mass (mg) to the 5 mg of tissue tested. Tests were performed using heart tissue from rabbits treated with vehicle and with Doxo.

	Tissue mass (mg)	***Protein aggregates-enriched fraction mass (mg)***
Control	5	0.285
Doxo	5	0.340

The SDS-PAGE profiles from Figure 12 demonstrated that this protocol was also applicable in New Zealand white rabbit's myocardial samples. Furthermore, these results showed that 5 mg of tissue seems to be enough to obtain a "clean" profile of aggregated proteins.

All the conditions tested are summarized in Figure 13.

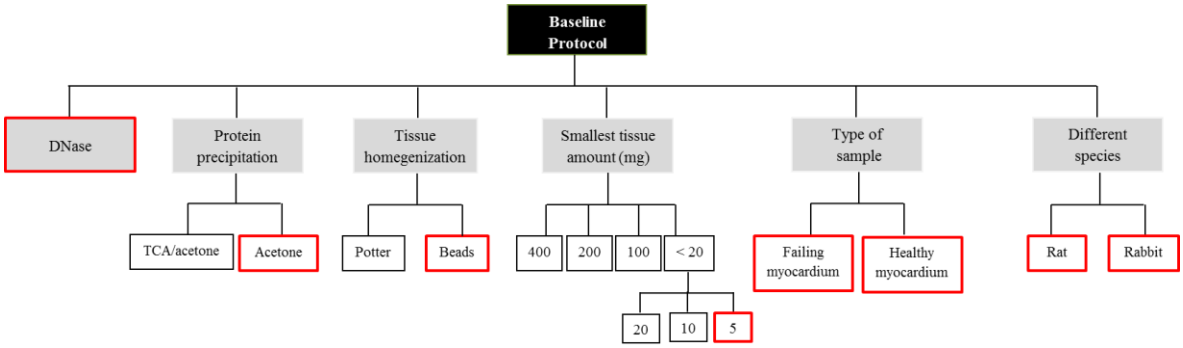

Figure 13: Overview of all experimental conditions tested over to baseline protocol. The optimized conditions are marked in red and are suitable for both animal models.

4.2 Optimized protocol for the isolation of protein aggregates-enriched fractions

After optimizing the methodology for protein aggregates enrichment it was possible to achieve a final protocol, described below. LV were defrosted, weighted and suspended in RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2.5% sodium deoxycholic, 1% NP40, 1 mM EDTA and protease inhibitor cocktail). Tissue homogenization was performed at a speed of 6500 rpm for 6 cycles of 30 seconds, using MagNA Lyser Instrument (Roche Diagnostics). Homogenates were centrifuged (16 060 x *g*, 5 minutes, 4ºC) to remove debris, organelles and particulates. The supernatants were incubated with DNase type I (NZYTech) for 15 minutes at 37ºC, followed by centrifugation (2 000 x *g*, 5 minutes). The protein concentration was determined using DC™ Protein Assay Kit (Bio-Rad). Protein samples were precipitated with ice-cold acetone overnight at -20ºC, followed by centrifugation at 14 000 x *g* for 15 minutes at 4ºC. Pellets were suspended in HEPES buffer (0.1 M HEPES, pH 7.4, 1% sarcosyl (w/v), 5 mM EDTA and protease inhibitor cocktail), and, subsequently, samples were centrifuged for 35 minutes at 100,000 x *g*, 4ºC. Detergent-insoluble fractions were collected and suspended in 30 µL SDS 10%, and an aliquot was taken to perform protein quantification. Proteins were suspended in SDS-PAGE loading buffer (0.5 M Tris-HCL pH 6.8, 50% glycerol, 10% SDS, bromophenol blue, 20% mercaptoethanol) and dissolved by heating for 10 minutes at 100ºC. Protein components of aggregate fractions were separated by 12% SDS-PAGE gel for 45 minutes at 200V. The resulted gel was stained with Coomassie Brilliant Blue G-250 (Bio-Rad) and visualized on ChemiDoc™ XRS Gel Imaging System (Bio-Rad).

4.3 Aging animal model

4.3.1 Characterization of the protein aggregates-enriched fractions isolated from left ventricle of young and aged rats

Protein aggregates-enriched fractions from LV of Young and Aged WKY rats were isolated and separated by SDS-PAGE (Figure 14).

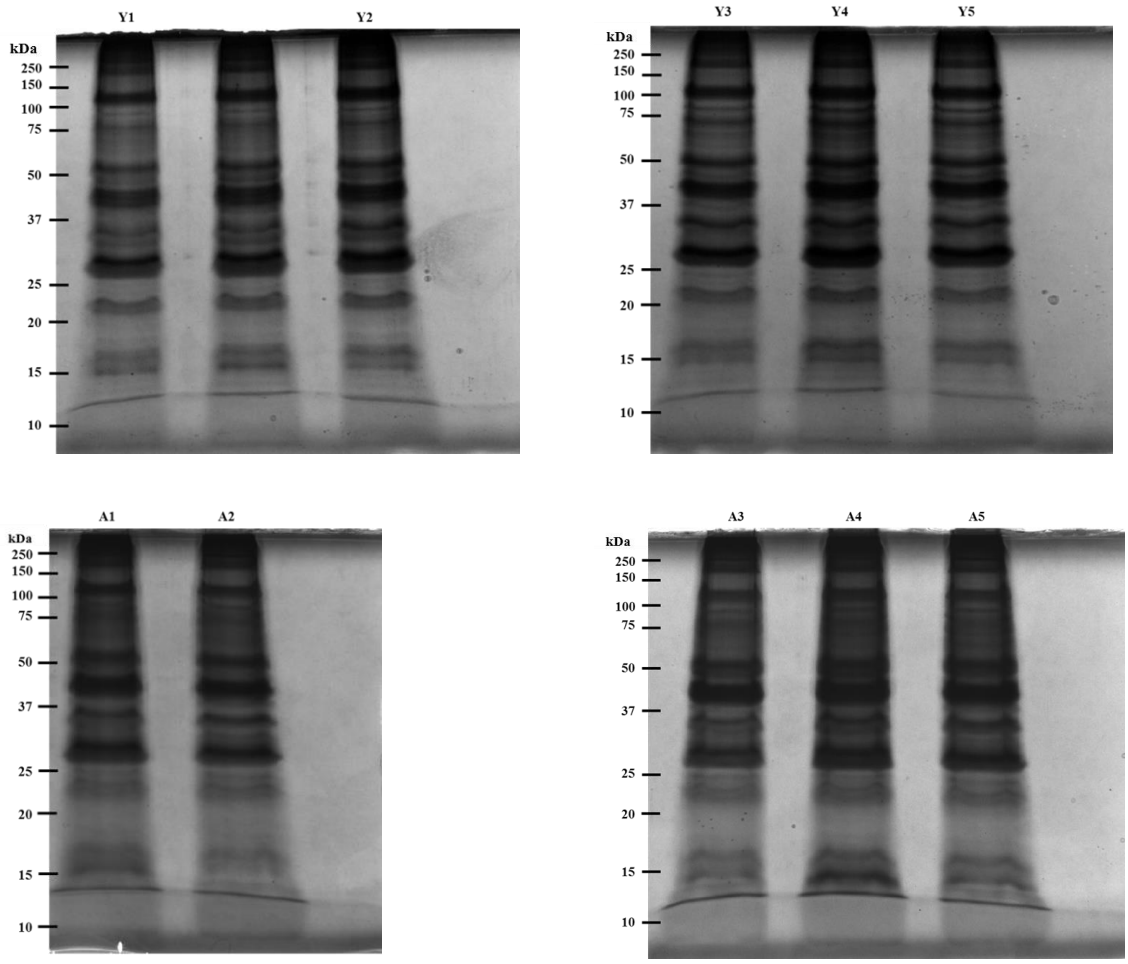

Figure 14: SDS-PAGE of the protein aggregates-enriched fractions obtained from LV of Young (Y) and Aged (A) WKY rats.

The band profiles obtained seem to be similar between Young and Aged rats. Furthermore, percentage of protein aggregates-enriched fractions was also determined and no significant differences were found between groups (Young=7.20±0.80 *vs* Aged=9.20±0.80, p=0.1151) (Figure 15).

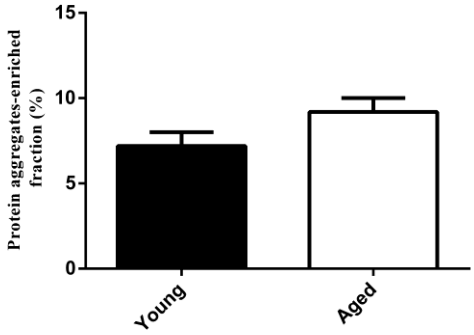

Figure 15: Percentage of the LV protein aggregates-enriched fractions determined for Young and Aged WKY rats. Values are represented by mean ± SEM. n=5 in each group.

4.3.2 Proteomic analysis after GeLC-MS/MS

Total protein aggregates-enriched fractions

In order to characterize protein aggregates-enriched fractions from Young and Aged rats myocardium, proteins from aggregates-enriched fractions were separated by SDS-PAGE, digested *in-gel* with trypsin and the peptides separated and analyzed by GeLC-MS/MS. This analysis allowed the identification of 2470 proteins with FDR<5%. After removing contaminants and select proteins with two or more peptides identified, a total of 1357 proteins were obtained.

The protein aggregates-enriched fractions were evaluated resulting in the identification of 1279 proteins in Young group and 1260 in Aged group. To characterize the exclusive aggregated proteins of each group, a Venn diagram was created using jvenn (Figure 16).

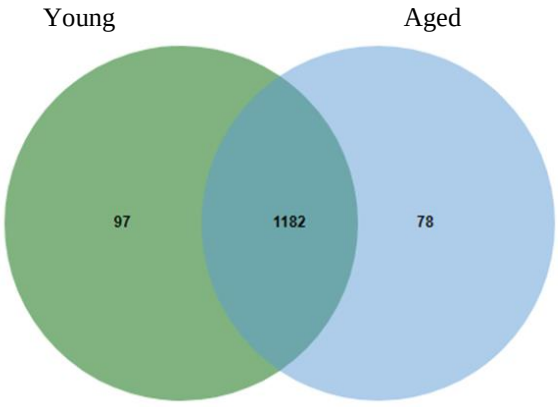

Figure 16: Venn diagram showing the distribution of protein aggregates-enriched fractions identified in Young and Aged groups (1279 and 1260, respectively): 1182 proteins are common in both groups; 97 proteins are exclusive of Young group and 78 proteins of Aged group.

As shown in Figure 16, 97 and 78 proteins were exclusively found in Young and Aged rats myocardium, respectively, which mean that about 87% of aggregated proteins are shared by both groups.

Hydropathy analysis

A property that strongly influences the aggregation propensity of a protein sequence is its hydrophobicity. In neurodegenerative diseases, proteins propensity for aggregation are more hydrophobic (102). Take that into account, we evaluate if the proteins that aggregate in heart tissue follow the same profile. We used the grand average of hydropathy (GRAVY) value as measure of the hydrophobicity of proteins. The GRAVY value, defined by the sum of hydropathy values of all amino acids divided by the protein length, was calculated for all aggregated proteins identified in Young and Aged groups (Figure 17).

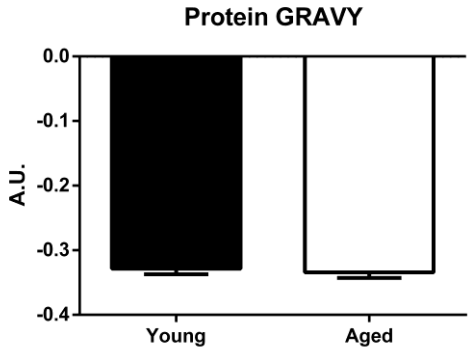

Figure 17: Protein GRAVY analysis. GRAVY value calculated for protein sequences identified in Young and Aged WKY rats. Values are represented by mean ± SEM.

This analysis revealed that hydropathy value does not change between groups (Young=-0.3286±0.0087; Aged=-0.3341±0.0088, p=0.6584), and that the majority of this aggregated proteins are hydrophilic, regarding that hydropathy value was negative.

Biological processes

With the purpose of ascertain the biological processes associated with proteins identified in both groups, ClueGO bioinformatic tool was used. For cluster analysis in ClueGO, we defined two protein groups - cluster 1 (marked by red color): aggregated proteins identified in Aged myocardium; cluster 2 (marked by green color): aggregated proteins identified in Young myocardium). Thus, a molecular network was created, to visualize which biological processes belong to each cluster (Figure 18).

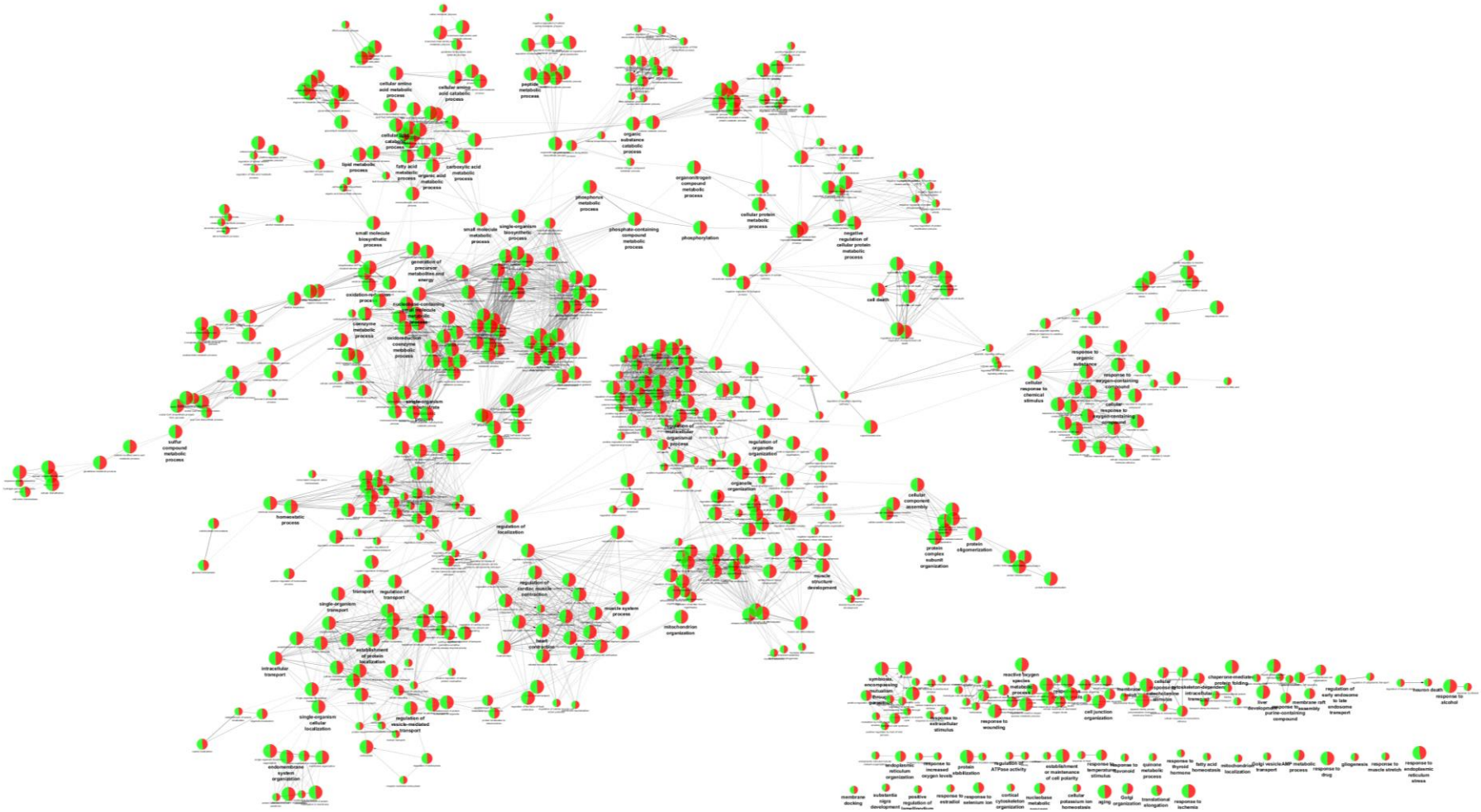

Figure 18: ClueGO network representing the main biological processes associated with aggregated proteins identified in Young and Aged rats myocardium. Each node represents a biological process and edges represent the connections between the nodes. Green part of each node represents the percentage of proteins identified in Young group that are related with that node biological process. Red part of each node represents the percentage of proteins identified in Aged group that are related with that biological process.

As observed in Figure 18, most of the biological processes are associated with both Young and Aged groups, since the green and red parts of the nodes are equal. However, biological processes such as homeostatic process, mitochondria organization, generation of precursor metabolites and energy, cell death, phosphorylation, intracellular transport, among others, are more representative of the Young group, which are represented by nodes with higher proportion of green. Likewise, regulation of cardiac muscle contraction, cardiac contraction, muscle structure development, organelle organization and its regulation, among others, are the most relevant in Aged group, being the proteins associated with these biological processes more predominant in this group.

Differential protein analysis

To evaluate the proteins that significantly differ in abundance in each group, a differential protein analysis was done taking into consideration the proteins identified in all rats (Figure 19).

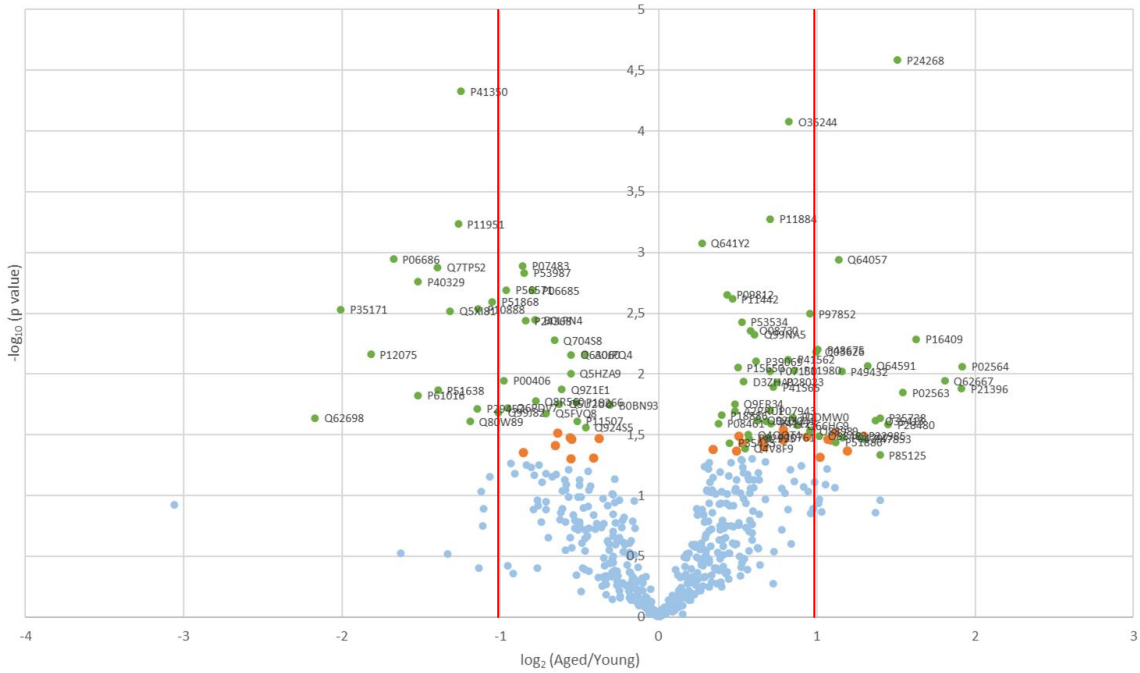

Figure 19: Volcano plot of all proteins identified with two or more peptides in 10 animals (5 Young *vs* 5 Aged rats). Each point represents a protein plotted as a function of $\log_2$ (fold-change: Aged/Young) and $-\log_{10}$ (*p*-value). Blue points correspond to the non-significantly deregulated proteins; orange points correspond to the non-consistently deregulated proteins; green points correspond to proteins consistently deregulated (represented by their UniProt KB code).

Differences in protein amounts between Young and Aged myocardium are represented according to their *p*-value and fold-change (Figure 19). Blue points represent the non-significantly deregulated proteins, whereas orange and green points represent statistically significantly deregulated proteins. But only proteins represented by green points passed the magnitude test, which means that these proteins are consistently deregulated in the group. Among these, only proteins with $\log_2$ (Aged/Young) <-1 or >+1 were considered differentially increased between the two groups. In this way, proteins with $\log_2$ (Aged/Young) less than -1 are markedly increased in Young group, and consequently, decreased in Aged group. Likewise, proteins with $\log_2$ (Aged/Young) greater than +1 are markedly increased in Aged group and conversely decreased in Young group. In this case, 15 and 18 proteins were significantly increased in Young and Aged groups, respectively, as described in Table 4.

Table 4: Full list of proteins (and their UniProt KB code) identified in aggregates-enriched fractions that were differentially increased in Young ($\log_2$ (Aged/Young) <-1) and Aged ($\log_2$ (Aged/Young) >+1) myocardium.

Proteins increased in Young group	Proteins increased in Aged group
P40329: Arginine--tRNA ligase, cytoplasmic	**Q64591:** 2,4-dienoyl-CoA reductase, mitochondrial
P51868: Calsequestrin-2	**P35738:** 2-oxoisovalerate dehydrogenase subunit beta, mitochondrial
Q7TP52: Carboxymethylenebutenolidase homolog	**Q64057:** Alpha-aminoadipic semialdehyde dehydrogenase
P61016: Cardiac phospholamban	**P21396:** Amine oxidase [flavin-containing] A
P41350: Caveolin-1	**P47853:** Biglycan
P51638: Caveolin-3	**P24268:** Cathepsin D
P10888: Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	**P85125:** Caveolae-associated protein 1
P12075: Cytochrome c oxidase subunit 5B, mitochondrial	**P04797:** Glyceraldehyde-3-phosphate dehydrogenase
P11951: Cytochrome c oxidase subunit 6C-2	**P51886:** Lumican
P35171: Cytochrome c oxidase subunit 7A2, mitochondrial	**Q62667:** Major vault protein
Q62698: Cytoplasmic dynein 1 light intermediate chain 2	**P16409:** Myosin light chain 3
Q5XI81: Fragile X mental retardation syndrome-related protein 1	**P02563:** Myosin-6
Q80W89: NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 11	**P02564:** Myosin-7
P29457: Serpin H1	**O88483:** [Pyruvate dehydrogenase [acetyl-transferring]]-phosphatase 1, mitochondrial
P06686: Sodium/potassium-transporting ATPase subunit alpha-2	**P49432:** Pyruvate dehydrogenase E1 component subunit beta, mitochondrial
	O35413: Sorbin and SH3 domain-containing protein 2
	P28480: T-complex protein 1 subunit alpha
	P22985: Xanthine dehydrogenase/oxidase

Human diseases associated with aggregated proteins

Analysis of proteins aggregates-enriched fractions from Young and Aged LV revealed that 78 proteins were exclusively aggregated in Aged hearts. In this way, to get more information about the biological relevance of these aggregated proteins, we investigated their association with human diseases. For this purpose, a Curated DisGeNET network was created (Figure 20).

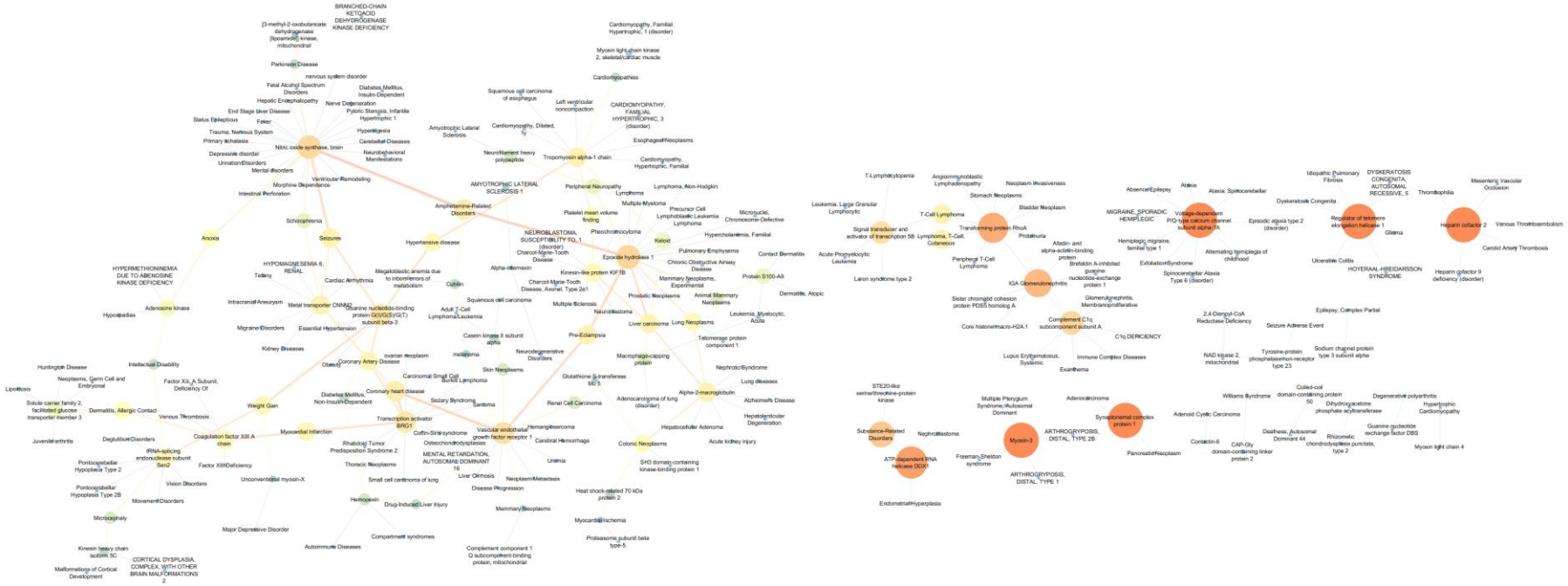

Figure 20: DisGeNET network representing the diseases associated with proteins exclusively aggregated in Aged group. Smaller nodes and those more blue correspond to proteins or diseases with few interactors. Larger nodes and those more orange correspond to proteins or diseases with more interactors.

This network provides information about which proteins are related to which human diseases. Several cardiovascular diseases, such as cardiomyopathy, myocardial infarction, coronary heart disease are associated with proteins that aggregate exclusively during aging, including tropomyosin alpha-1 chain, transcription activator BRG1 and vascular endothelial growth factor receptor 1. These findings suggest a possible involvement of these proteins in the pathophysiology of cardiac aging.

4.4 Doxo-induced cardiotoxicity animal model

4.4.1 Characterization of the protein aggregates-enriched fractions isolated from left ventricle of Control and Doxo-treated rabbits

Protein aggregates-enriched fractions from the LV of New Zealand rabbits were also isolated and separated by SDS-PAGE (Figure 21), according the optimized protocol.

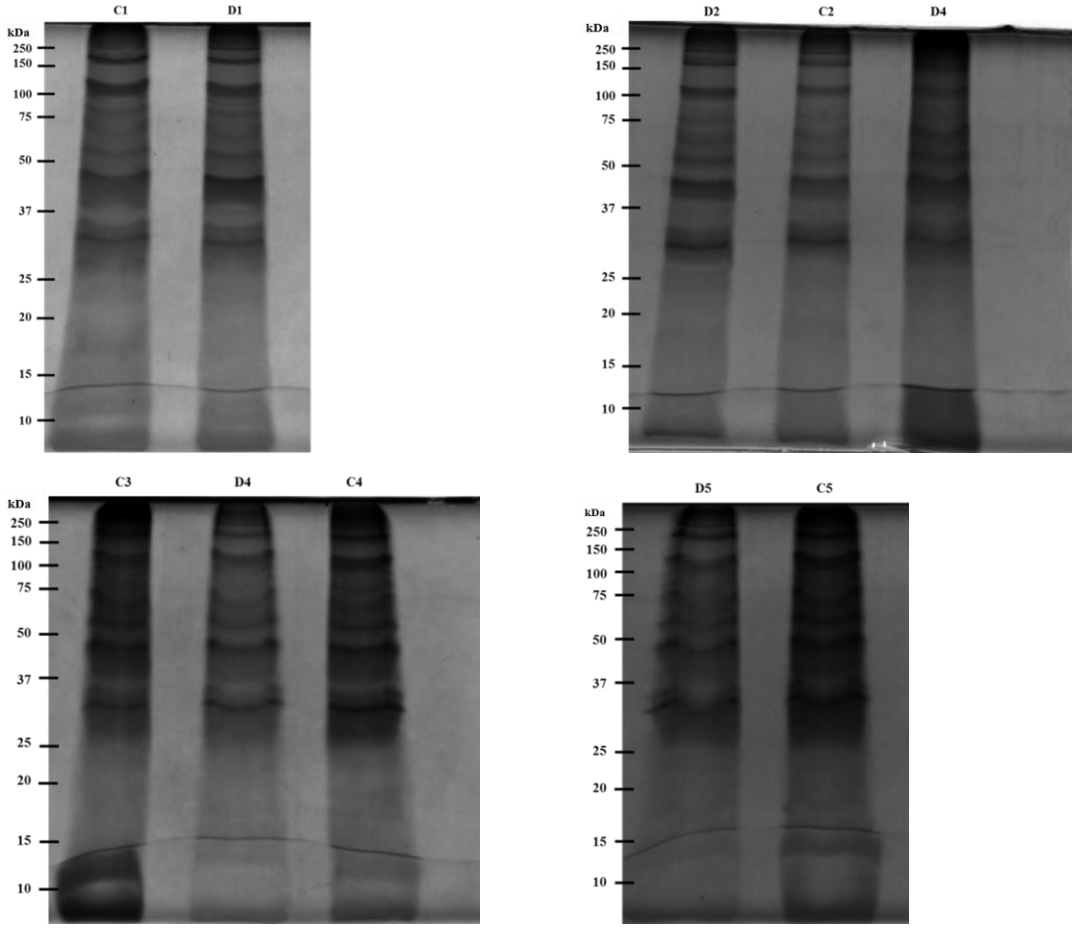

Figure 21: SDS-PAGE of the protein aggregates-enriched fractions obtained from LV of Control (C) and Doxo-treated (D) rabbits.

SDS-PAGE profiles, observed in Figure 21, were similar in Control and Doxo-treated animals. Similarly to the aging model, no significant differences were found in the percentage of protein aggregates-enriched fractions between groups (Control=9.400±1.691; Doxo=9.600±2.272, *p*=0.9454) (Figure 22).

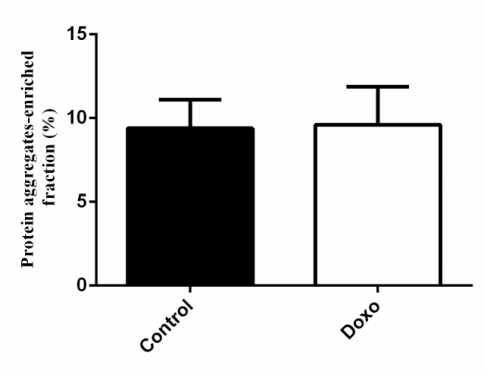

Figure 22: Percentage of the LV protein aggregates-enriched fraction determined for Control and Doxo-treated rabbits. Values are represented by mean ± SEM. n=5 in each group.

4.4.2 Proteomic analysis after GeLC-MS/MS

Total protein aggregates-enriched fractions

Identification of proteins present in aggregates-enriched fractions from Control and Doxo-treated rabbits was performed by GeLC-MS/MS, allowing the identification of 661 proteins with FDR<5%. After removing contaminants and select proteins with two or more peptides identified, a total of 287 proteins were obtained.

The total protein aggregates-enriched fractions were evaluated resulting in the identification of 274 proteins in Control group and 267 in Doxo group. Then, a Venn diagram was created to characterize the aggregated proteins exclusive of each group (Figure 23).

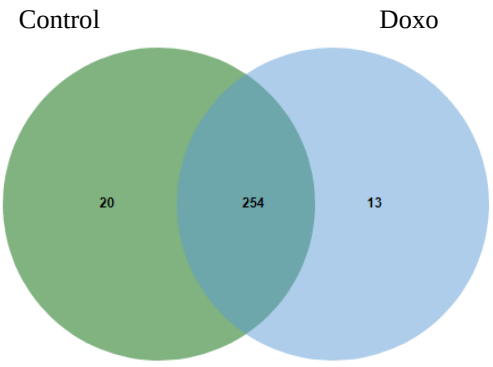

Figure 23: Venn diagram showing the distribution of protein aggregates-enriched fractions identified in Control and Doxo groups (274 and 267, respectively): 254 proteins are common in both groups; 20 proteins are exclusive of Control group and 13 proteins of Doxo group.

As shown in Figure 23, 20 and 13 proteins were exclusively found in Control and Doxo-treated rabbits, respectively, which mean that about 89% of aggregated proteins are shared by both groups.

Hydropathy analysis

To infer about the hydrophobicity or hydrophilicity of the proteins, GRAVY was calculated for all aggregated proteins identified in Control and Doxo-treated rabbits (Figure 24).

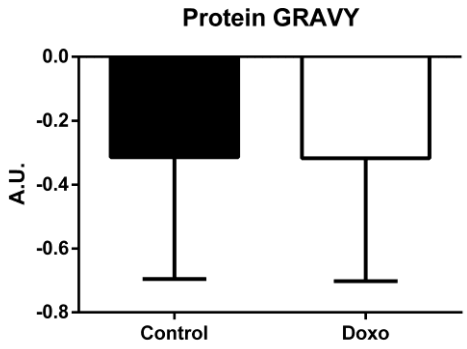

Figure 24: Protein GRAVY analysis. GRAVY value calculated for protein sequences identified in Control and Doxo-treated rabbits. Values are represented by mean ± SEM.

This analysis revealed that hydropathy value is similar between groups (Control=-0.3143±0.0230; Doxo=-0.3177±0.0235, p=0.9192), and that the majority of this aggregated proteins are hydrophilic.

Biological processes

For cluster analysis in ClueGO, we used the two protein groups - cluster 1 (marked by red color): aggregated proteins identified in Doxo myocardium; cluster 2 (marked by green color): aggregated proteins identified in Control myocardium. Regarding molecular network, only few GO annotations/terms were visualized because the database for the *Oryctolagus cuniculus* species was from 2014 terms (data not shown). Consequently, BLAST analysis was performed for sequence alignment with proteins from *Homo sapiens*, being selected proteins with ≥95% of homology. Then, a molecular network was constructed accordingly to the biological processes associated with identified proteins (Figure 25).

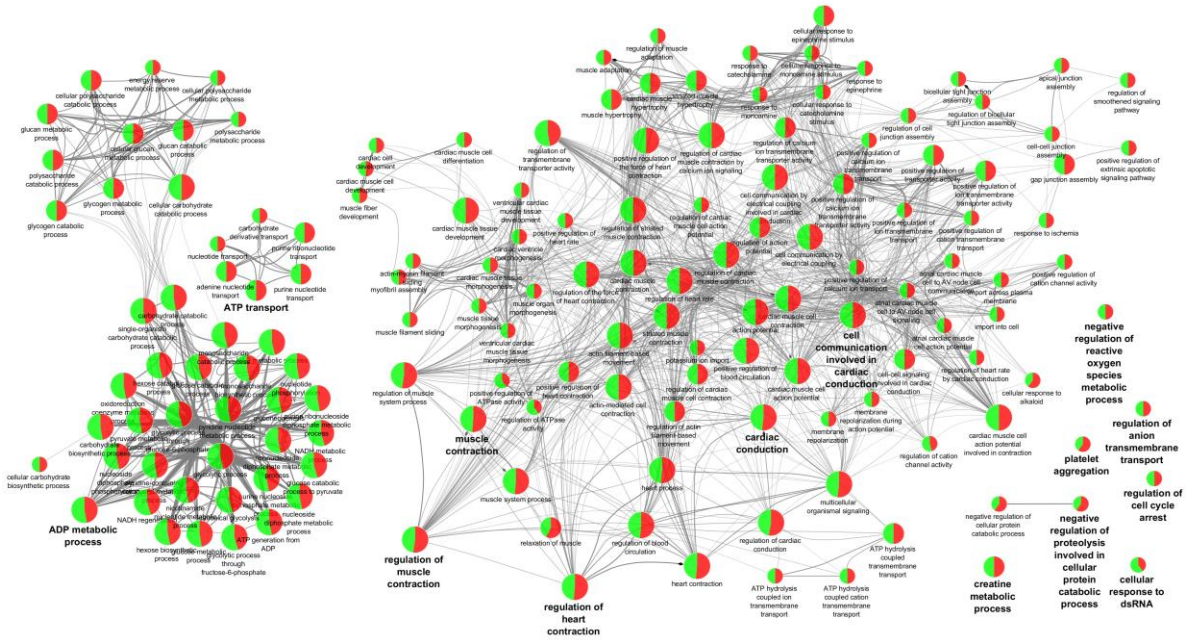

Figure 25: ClueGO network representing the main biological processes associated with aggregated proteins identified in Control and Doxo-treated rabbits' myocardium. Each node represents a biological process and edges represent the connections between the nodes. Green part of each node represents the percentage of proteins identified in Control group that are related with that biological process. Red part of each node represents the percentage of proteins identified in Doxo group that are related with that biological process.

As observed in Figure 25, most of the biological processes are associated with both Control and Doxo groups, since the green and red parts of the nodes are equal. However, biological processes such as ADP metabolic process are representative of the Control group, since these nodes have higher proportion of green. Likewise, regulation of cardiac contraction, cardiac conduction, platelet aggregation, among others, are representative of the Doxo group, since these biological processes have higher percentage of proteins identified in Doxo group.

Differential protein analysis

To evaluate the proteins that are markedly increased or decreased in both groups, a differential protein analysis was done considering the proteins identified in all rabbits (Figure 26).

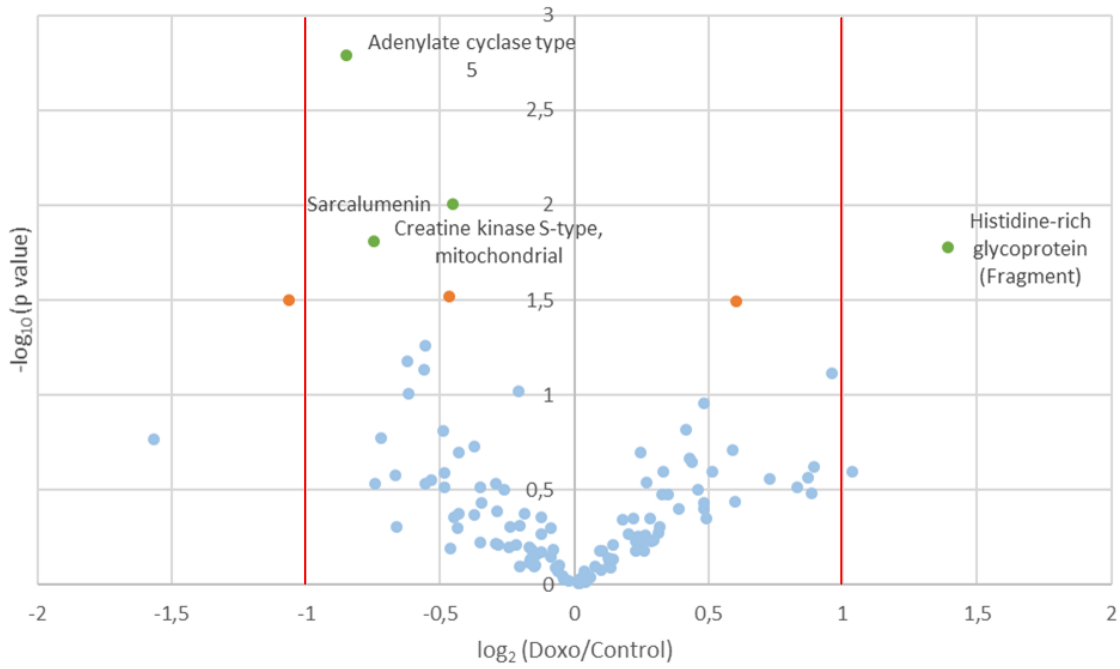

Figure 26: Volcano plot of all proteins identified with two or more peptides in 10 animals (5 Control *vs* 5 Doxo-treated rabbits). Each point represents a protein plotted as a function of $\log_2$ (fold change: Doxo/Control) and $-\log_{10}$ (*p*-value). Blue points correspond to the non-significantly deregulated proteins; orange points correspond to the non-consistently deregulated proteins; green points correspond to proteins consistently deregulated (represented by their UniProt KB code).

Proteins represented by green points with $\log_2$ (Doxo/Control) <-1 or >+1 were considered differentially deregulated between the two groups. In this way, proteins with $\log_2$ (Doxo/Control) less than -1 are markedly increased in Control group, and consequently, decreased in Doxo group. Likewise, proteins with $\log_2$ (Doxo/Control) greater than +1 are markedly increased in Doxo group, and conversely, decreased in Control group. In this case, only histidine-rich glycoprotein showed to be increased in Doxo group.

Human diseases associated with aggregated proteins

Analysis of aggregates-enriched fractions from Control and Doxo hearts revealed that 13 proteins were exclusively aggregated in Doxo-treated animals. In this way, a Curated DisGeNET network was created, in order to evaluate the association between exclusively aggregated proteins in Doxo group with human diseases (Figure 27).

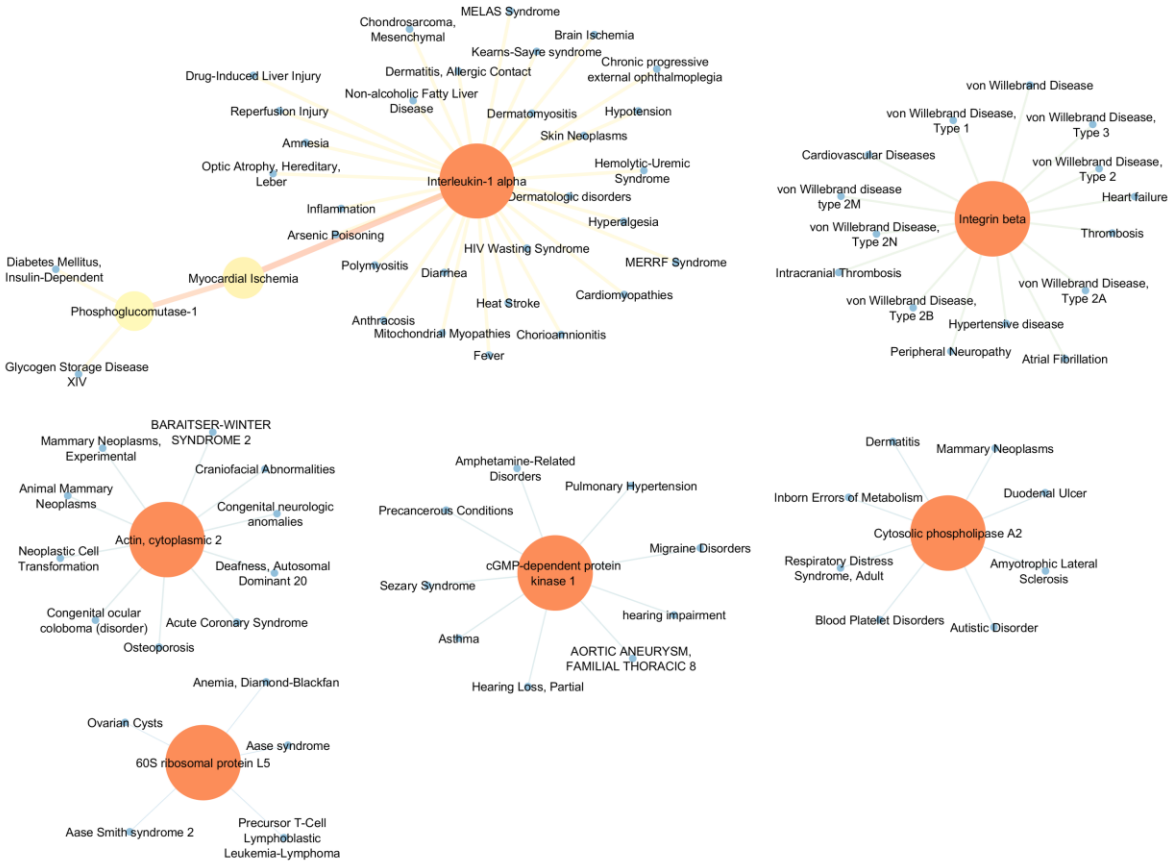

Figure 27: DisGeNET network representing the diseases associated with proteins exclusively aggregated in Doxo group. Smaller nodes and those more blue correspond to proteins or diseases with few interactors. Larger nodes and those more orange correspond to proteins or diseases with more interactors.

As expected, several cardiovascular diseases, such as cardiomyopathies, myocardial ischemia and HF, are associated with proteins that aggregate exclusively after Doxo treatment, suggesting a possible involvement of these proteins in Doxo-induced cardiotoxicity.

5. DISCUSSION

5.1 Methodological optimization for the isolation and characterization of the protein aggregates-enriched fractions

The first aim of this thesis was to optimize the methodology for the isolation and characterization of compact protein aggregates from cardiac tissue from rats and rabbits, based on the method described by Ayyadevara *et al.* (18). Our concern was to report all conditions tested during the experimental implementation. Therefore, two steps were added over Ayyadevara *et al.* (18) protocol: degradation of genomic DNA with a DNase treatment and protein purification by precipitation with TCA/acetone. In fact, after protein extraction, removal of all types of contaminants such as detergents, lipids and nucleic acids is essential, since they may interfere with downstream applications (100). The visualization of the resulting gel revealed no differences between the SDS-PAGE profiles of the sample treated with TCA/acetone comparing to the non-treated sample. This protein precipitation method is often used, however it presented an apparent disadvantage due to the probable protein loss during the procedure, caused by incomplete solubilization of the pellets and the acetone washing step (103). Since TCA is a strong acid, the resulting pellet was usually hard to dissolve (104), being extremely time-consuming, so in the subsequent tests this method was removed. Additionally, the poor intensity of the bands observed in the gel was possibly due to the fact not all sample was properly loaded into the gel (since the pellet was hard to dissolve) or may indicate that a higher amount of sample (>15 mg) is required to achieve a better protein aggregates-enriched fractions profile.

In the second test, two distinct amounts of LV from different animal models were tested. Based on gel analysis it was possible to obtain protein aggregates-enriched fractions, thereby demonstrating that the methodology worked both in healthy and failing myocardial samples. Interestingly, the SDS-PAGE profile showed that lane A displays higher amounts of aggregates comparing to lane B, despite starting with smaller amount of tissue. These differences may be due to sample type, it is expected that failing hearts present more protein aggregates since protein quality control can be already compromised. The third test aimed to improve the purity and, consequently, to avoid sample dragging later on the SDS-PAGE profiles. To achieve this, we used protein precipitation method with ice-cold acetone. This method has the advantage of being simple to perform and removing interfering species, such as lipids, genomic DNA and detergents, which proved to maximize the recovery of proteins in small samples. These findings are in agreement with a previous study performed in brain tissue samples, in which protein precipitation using acetone resulted in a greater protein recovery and in less time to solubilize the protein pellet, comparing to TCA/acetone method (103). Since the amount of LV available is very limited in the aging animal model, it was essential to introduce this step in the protocol, in order to obtain

maximal protein aggregates-enriched fractions recovery from minimal tissue amounts, such as 10 mg of tissue.

Obtaining high quality and high yield of proteins is an important factor for further downstream protein characterization methods, such as in SDS-PAGE and MS (100). In fact, protein yield depends on the type of homogenization method selected (105, 106). Thus, in a fifth experiment, other homogenizing method using a beads system was tested and compared to the previously potter-based mechanical homogenization. Beads homogenizer proved to be a more promising methodology, because the protein yield obtained from the same amount of tissue was always higher compared to potter homogenizer. With this protein extraction method, 5 mg of tissue was enough to obtain a "clean" profile of the protein aggregates-enriched fractions, thereby solving the problem of limited amount of tissue. Therefore, beads homogenizer is an efficient method, which ensures efficient disruption, homogenization, as well as higher yields of proteins comparing with potter homogenizer.

According to Aye *et al.* (107) work, more than 2200 LV proteins could be identified, with a FDR <1% through trypsin-based in-gel digestion followed by GeLC-MS/MS analysis. In such study, 50 µg of proteins were loaded into the gels, thus this value served as reference for this study. Taking this into account, the purpose of the sixth test was to determine if the amount of proteins loaded into the gel was satisfactory to perform a GeLC-MS/MS analysis. Furthermore, the impact of protein precipitation with acetone on the aggregated proteins yield was evaluated. Firstly, results demonstrated reproducibility of the optimized method, because the SDS-PAGE profiles were similar to previous tests using the same amount and type of sample. In fact, the use of acetone as precipitation method improved protein extraction yield of protein aggregates-enriched fractions, relative to the same amount of tissue, in both failing and healthy myocardial samples. Furthermore, treatment with acetone seems to be important to preserve low molecular weight proteins. We were also able to show that 5 mg of tissue is enough to obtain 10 times more protein aggregates-enriched fraction than the amount necessary to perform a GeLC-MS/MS with trypsin *in-gel* digestion (107). This amount of tissue displayed a good definition of the bands and not a trawling, as observed for the double amount of tissue (10 mg). Moreover, the resulting pellet was smaller and the solubilization was easier, avoiding sample loss in this step.

After optimizing the conditions for aggregates enrichment, the last experiment aimed to test if this method was also applicable in a different animal species other than rat, *Oryctolagus cuniculus,* i.e. New Zealand white rabbits. Both animals that received vehicle or Doxo were analysed showing a "clean" SDS-PAGE profile, suitable for studying myocardial protein aggregates in this species. Again 5 mg of tissue provided a good band definition and was enough to obtain more than 50 µg of proteins, reaching the goal defined in the study of Aye *et al.* (107)

5.2 Proteomic analysis of protein aggregates-enriched fractions

Defects on protein quality control promote loss of protein homeostasis, which leads to accumulation of misfolded proteins and protein aggregates, with consequent toxic effects to the cells. Impaired proteostasis is particularly detrimental to cardiomyocytes since they have restrictive regenerative capacity (54). Therefore, maintaining balanced protein quality control is fundamental to minimize cellular dysfunction and death (2). For this reason, the identification of aggregated proteins provides a starting point to understand their influence in collapse of protein homeostasis in both aging and cardiotoxicity conditions. Regarding methodological approach used in the present work, it is important to mention that protein aggregates are insoluble in sarcosyl (18), thus, information derived from soluble aggregates might have been disregarded.

Cardiovascular impact of aging in protein aggregation

As mentioned before, during physiological aging protein homeostasis gradually becomes compromised and several proteins tend to aggregate (108). Previous study demonstrated that hearts from aged mice showed an increase in the number and abundance of aggregated protein components, relative to those from young-adult mice (18). Surprisingly, patterns and intensities of the bands as well as the percentage of protein aggregates-enriched fractions found in our work were similar between Young and Aged hearts (supplement A). So, despite no differences were observed in the relative amount of protein aggregates-enriched fractions between both groups, the type of proteins present in those fractions are associated to distinct physiologic roles in this specific animal model. Thus, as expected, we found differences in protein constituents of age-derived aggregates. In fact, GeLC-MS/MS analysis of protein aggregates provides identification of distinct proteins in Young and Aged groups, being 97 and 78 proteins exclusively identified in Young and Aged hearts, respectively, suggesting that aging promotes aggregation of different proteins when compared to their young counterparts. Nowadays, it is well known that neurodegenerative diseases, such as Alzheimer's disease, are intimately related to protein aggregation, which is involved in their pathophysiology. The protein aggregate, in this neurodegenerative disease, is predominantly constituted by a higher amount of hydrophobic proteins, which are more susceptible to aggregation (102). However, in our study we did not find the same profile of proteins in the LV tissue. In fact, GRAVY analysis revealed no differences between the two groups and mostly of the proteins found in the aggregates from both groups were in general more hydrophilic. This finding suggests that protein aggregation pattern in cardiac tissue may differ from that in nervous tissue, namely in intensity, mechanisms underlying aggregation and proteins heterogeneity.

Nowadays, bioinformatic analysis provide researchers the possibility to have insights into the specific biological processes associated with proteins identified in different conditions, such as the ones observed in protein aggregates-enriched fractions. Therefore, it is useful to pinpoint potentially deregulated biological processes that occur during the natural course of aging. Most of the biological processes presented in the ClueGO network are equally shared by the two groups, observed by the equivalent percentage of green (Young group) and red (Aged group) parts in several nodes. Indeed, this outcome was expected since 87% of total protein aggregates-enriched fractions were common to both groups. Nevertheless, there were specific biological processes such as homeostatic process, mitochondria organization, generation of precursor metabolites and energy, cell death, representative of the Young group, suggesting that protein aggregation is a normal physiological event that plays an important role in the maintenance of cardiac structure and function even under healthy conditions. In fact, aggregated proteins associated with mitochondria organization, which includes mitochondrial morphogenesis and distribution, replication of the mitochondrial genome and synthesis of new mitochondrial components, may be due to the increase of mitochondria biogenesis in Young hearts when compared to Aged hearts (109-111). Thus, the protein aggregates present in Young group could be linked to the more efficient/higher protein turnover (112) that occurs at this age, therefore representing a vital regulatory/basal mechanism for the elimination of damaged proteins or organelles. On the other hand, we also noticed that specific biological processes are more representative of the Aged group, which include regulation of cardiac muscle contraction, cardiac contraction, organelle organization and regulation, among others. The presence of proteins involved in cardiac contraction in aggregates from aged hearts has already been reported (18). This finding may possibly suggest that the dysfunction with posterior accumulation of these proteins can contribute to cardiac contractility impairment observed during the natural process of aging. At the same time, aging has been related with modifications in structure and organization of several organelles, namely mitochondria (113), which support our results, taking into account that proteins involved in this biological process were more abundant in aggregates from Aged hearts.

Differential protein analysis of aggregates revealed 33 proteins that significantly differ in abundance between Young and Aged hearts, from which 18 proteins were markedly increased with age. Cardiac contractility and relaxation are regulated by Ca^{2+} homeostasis. Several proteins present in SR, such as Ca^{2+}-ATPase (SERCA), phospholamban, calsequestrin-2 and ryanodine receptors (RyR), play a key role in regulation of intracellular Ca^{2+}-handling. SERCA is responsible for the sequestration of cytosolic Ca^{2+} into SR lumen, being regulated by phospholamban. In this way, dephosphorylated phospholamban can interact with SERCA, decreasing its pumping activity, whereas phosphorylated phospholamban increase affinity of SERCA for Ca^{2+}. On the other hand,

calsequestrin-2 is the major Ca^{2+}-binding protein in cardiomyocytes, playing an important function in Ca^{2+} storage and in regulation of Ca^{2+} release from SR into cytosol via RyR (114). These two proteins, phospholamban and calsequestrin-2, were markedly increased in protein aggregates from Young group. Moreover, sodium/potassium-transporting ATPase (Na^+/K^+-ATPase) subunit alpha-2 was also increased in Young hearts. Na^+/K^+-ATPase is a transmembrane protein that catalyzes the active transport of Na^+ and K^+ across the plasma membrane, thereby regulating the membrane potential, creating a Na^+ transmembrane gradient and, indirectly, cardiac contractility. This protein is composed by distinct isoforms: catalytic α-subunit isoform (α1, α2 or α3) and β-subunit isoform (β1 or β2). Na^+/K^+-ATPase isoforms play a significant role in regulating myocardial function during development stages, as well as in pathological conditions, like hypertension. Interestingly, during postnatal maturation, the α3 isoform is replaced by α2 isoform, presenting higher expression and activity of this isoform in adult rat heart (115). The higher abundance of phospholamban, calsequestrin-2 and Na^+/K^+-ATPase isoform α2 in aggregates from Young hearts may suggest a higher turnover/renovation of these proteins in order to maintain cardiomyocytes physiological demands of the faster, healthier and more contractile hearts of the Young group (116).

Cardiac muscle requires considerable amounts of ATP daily, which is supplied mainly by mitochondrial oxidative phosphorylation, to sustain normal contractile activity. So, balancing mitochondrial biogenesis and removal of damaged mitochondria by mitophagy is crucial for keeping a healthy population of mitochondria, ATP production and cellular homeostasis. However, several factors, such as aging, failure to preserve the critical balance between mitochondrial biogenesis and mitophagy, leading to an increase of dysfunctional mitochondria that contribute to several diseases (60, 109-111). Aggregates from Young hearts revealed higher abundance in mitochondrial proteins, more specifically in cytochrome c oxidase of the mitochondrial respiratory chain, which is the terminal enzyme complex of the mitochondrial electron transport chain. These findings can be explained by the increase in mitochondrial biogenesis to support the elevated energy requirements of the young cardiomyocytes. Thus aggregation of these proteins is possibly due to the higher protein renewal at this age that slows down with aging.

Among the most abundant proteins in the aggregates from Aged group we also found mitochondrial proteins, such as monoamine oxidase (MAO). As mentioned before, oxidative stress is one of the factors involved in pathophysiology of HF and cardiac aging (17, 58). Among the sources of oxidative stress, the mitochondrial enzymes MAO has been proposed to be a great contributor to ROS production in the heart. These enzymes, that exist in two isoforms MAO-A and MAO-B, are responsible for oxidative deamination of neurotransmitters and exogenous amines, resulting in H_2O_2 production (117, 118). Our results showed that MAO-A is increased in aggregates from aged hearts. Interestingly, MAO-A activity is increased in rat models of HF and

cardiac aging (117, 119), as well as MAO-A-dependent H_2O_2 production, suggesting that this enzyme is one of the major factors involved in cardiac oxidative stress during aging (117). Villeneuve *et al.* showed that increasing MAO-A expression, to pathophysiological levels observed in failing and aging hearts, trigger deleterious effects in the heart such as oxidative stress increase, mitochondrial damage and peroxisome proliferator-activated receptor-c coactivator-1 α (PGC-1α) downregulation (a master regulator of mitochondrial biogenesis), contributing to cardiomyocyte necrosis and HF (119). Moreover, MAO-A-dependent ROS production disrupts autophagic flux, leading to dysfunctional mitochondria accumulation and cell death (118). Interestingly, we observed that MAO-A aggregation was increased in aged hearts, that could be seen as a protective mechanism, to avoid MAO-A-dependent ROS production deleterious effects, and therefore the cell, probably, try to remove/eliminate this protein through aggregation. Furthermore, MAO-A protein levels seem to be regulated/degraded by UPS in certain regions of the brain (120). Also, it is known that protein degradation systems activity, such as UPS, decline with aging. In this way, the increase of this protein in aggregates from aged hearts may also suggest an impairment of protein degradation via UPS, which cause MAO-A aggregation. Therefore, more studies are needed in order to characterize the role of MAO-A in mechanisms underlying cardiac aging, and investigate this protein as potential target for the treatment of age-associated cardiovascular diseases.

Proteins related to contractile function, such as myosin-6 (α-myosin heavy chain), myosin-7 (β-myosin heavy chain) and myosin light chain 3, were also increased in protein aggregates from Aged hearts. Myosin, exist as two heavy chains and four light chains, exhibiting a pivotal role in the regulation of cardiac contraction. Correct myosin folding and assembly are essential for the maintenance of sarcomere structure, and thereby crucial for its proper function (121). These processes require multiple factors, including HSP70 and HSP90 chaperones (122). However, aging can interfere with protein folding and refolding as well as with protein degradation systems, resulting in the aggregation of unfolded or misfolded proteins (18, 41). In fact, a decline in the induction of several chaperones and proteasome activity, which is involved in myosin degradation (123), during aging has been reported (64, 65, 67-69). Thus, the greater amount of myosin in aggregates from aged hearts may suggest a dysfunction of these proteins, possibly due to the folding or degradation impairment, that can contribute to the decreased contractility during cardiac aging.

As mentioned before, chaperones are essential for proteins to acquire their native structure, by assisting protein folding, and therefore they have a crucial role in preventing protein aggregation. Chaperonin containing TCP1 complex (CCT), also known as the TCP1 ring complex (TRiC), is responsible for the folding of approximately 10% of proteins (4, 45). One of those proteins is actin, whose correct folding and prevention of aggregation are essential for the

formation of thin filaments, contributing to proper function of the sarcomere (124). T-complex protein 1 subunit alpha, a molecular chaperone that is a member of TRiC chaperonin, showed to be increased in aggregates from aged hearts. Thus, we hypothesized that aggregation of this chaperonin may suggest its dysfunction in aging with effects in myofilamentary proteins folding, that can underlie cardiac contractility impairment observed in aging. Interestingly, TRiC chaperonin seems to be important to restrain the toxic aggregates accumulation in Huntington's disease, suggesting a protective role of this complex, at least in brain cells (125). Therefore, TRiC can be a new target for future studies, to elucidate its role in cardiac aggregates suppression and its implication in myofilamentary proteins function during aging. Moreover, cardiomyocytes hypertrophy, accompanied by increased myofilamentary density, is naturally associated to aging and may contribute to the accumulation of important structural myofilamentary proteins, such as myosin, in aggregates from Aged rats (126).

Cathepsin D is another protein that was increased in aged hearts. This protein is the main aspartic protease responsible for protein degradation within lysosomes. Any defects in lysosomes, and consequently, autophagy machinery lead to several diseases, including neurodegenerative and aging-related diseases, as a result of protein misfolded accumulation (127, 128). Wu *et al.* showed that cathepsin D deficiency in mice hearts caused impairment of myocardial autophagosome removal, evidencing its role in cardiac autophagic flux. Also, the lack of this protein promotes the development of restrictive cardiomyopathy (128). Furthermore, it has been reported that cathepsin D is the main lysosomal enzyme involved in α-synuclein degradation, implicated in Parkinson's disease pathogenesis, and therefore protects against α-synuclein aggregates toxicity (127). Thus, up regulation of cathepsin D in aggregates from aged hearts may indicate dysfunctional protein-clearance machinery. Proteolytic systems activities, namely autophagy, are known to decrease during aging, so aggregation of this protein can be one of the contributors to this decline. All these findings make cathepsin D an interesting target for further studies.

Major vault protein (MVP) is the major component of the vault particle, whose functions and regulation are not well elucidated, although it appears to be involved in multi-drug resistance. This protein also seems to have an important role in cell survival, possibly mediated by post-translational modifications in MVP, which promote an inhibition of autophagy-mediated cell death (129). The higher abundance of aggregated MVP in aged hearts may possibly be implicated in increase of cell death during aging. Since the functions and regulatory mechanisms of MVP are not fully understood, this protein may represent a potential new target for research in cardiovascular pathophysiology.

Cardiac aging is accompanied by an increase in fibrosis (collagen content), one of the major contributors to progressive increase in ventricular stiffness and impaired diastolic function

(130). Lumican, a protein that binds collagen and facilitates collagen assembly, was markedly increased in Aged aggregates hearts. Previous work demonstrated that the lack of lumican contributes to fibrosis, showing the protective role of this protein in pathogenesis of cardiac fibrosis, and evidencing its potential role as novel target for cardiac fibrosis therapy (131). Thus, the aggregation of lumican may contribute to the increase in fibrosis observed in aged hearts (130). Further studies are required to infer about the implication of lumican aggregation during cardiac aging.

The correct functioning of the cells depends on effective proteostasis. Decline in proteostasis mechanisms led to protein misfolding and aggregation, which in turn can cause a broad range of diseases (1, 6). Indeed, aging *per se* represents a relevant cardiovascular risk factor. Thus, it is expected that age-related impairment of proteostasis in cardiomyocytes play a role in the development of several cardiovascular diseases (3, 18, 63). In fact, DisGeNET analysis revealed that several proteins exclusively identified in aggregates from Aged hearts are involved in many diseases, namely in cardiovascular and neurological diseases. For example, α-2 macroglobulin is associated with Alzheimer's disease, showing a key role in the inhibition of amyloid formation (132). This finding may suggest a common feature/link between protein aggregation in the brain and in the heart during aging. Several other proteins, such as transcription activator BRG1, vascular endothelial growth factor receptor 1, heat shock related 70kDa protein 2 and proteasome subunit beta type-5 are associated with several cardiovascular diseases, like myocardial infarction, coronary heart disease and myocardial ischemia. Thus, aggregation of these proteins during normal aging process may contribute to the pathophysiology of various cardiovascular diseases.

Cardiovascular impact of Doxo-induced cardiotoxicity in protein aggregation

Cardiotoxicity is capable to induce changes in proteome of adult cardiomyocytes, namely in proteins involved in apoptosis, energy metabolism, stress response and different signaling pathways (22). In addition, Doxo also promotes modifications in components of protein quality control, being associated with proteostasis impairment and protein aggregation (48, 85, 90). Patterns of bands of protein aggregates-enriched fractions were similar between Control and Doxo-treated rabbits. Also, no differences were observed in bands intensities of the gel and in the percentage of protein aggregates-enriched fractions between the two groups (supplement B). It would be expected that Doxo-treated animals will have higher amounts of protein aggregates in comparison to Controls, since its described effects on a variety of different proteins modifications/dysfunction, including the ones regulating proteostasis. This unexpected result can

be due to the fact that these animals do not yet presented the phenotype typically associated to Doxo-induced cardiotoxicity, namely LV dilation, reduced ejection fraction (EF) and contractile dysfunction (133). In fact, echocardiographic evaluation showed that Doxo-treated animals, comparing to the control animals, presented concentric hypertrophic hearts with preserved EF, suggesting that these animals are in an early stage of Doxo-induced cardiotoxicity, and consequently, the proteostasis is not yet compromised (data not shown). We believe that the fact that cardiac functional evaluation and also tissue collection were carried out 1 week after Doxo terminus injection was not sufficient to observe the chronic cardiotoxicity molecular effects induced by Doxo. However, there are already changes at the cellular level, including higher amount of fibrosis and cardiomyocytes hypertrophy (data not shown).

Despite the similarity between SDS-PAGE profile and amount of protein aggregates in both groups, differences may reside in protein content. GeLC-MS/MS analysis provides the identification of distinct proteins in both groups. 20 and 13 proteins were exclusively identified in Control and Doxo hearts, respectively, which suggests that Doxo may have specific effects on particular proteins that can promote its cardiotoxicity. GRAVY analysis of aggregated proteins from Control and Doxo hearts revealed no differences between the two groups, being generally more hydrophilic. This finding corroborates our hypothesis that protein aggregation pattern may be different in cardiac tissue, when compared to nervous tissue, since in neurodegenerative diseases proteins prone to aggregation are more hydrophobic (102).

ClueGO analysis provided a general view over the main biological processes associated with proteins found in LV from both Control and Doxo-treated rabbits. Most of the biological processes presented in the ClueGO network are equally shared by the two groups, observed by the equivalent percentage of green (Control group) and red (Doxo group) parts in several nodes. This outcome would be expected since about 89% of aggregate proteome is common to both groups. However, there were some biological processes specific for each group, such as ADP metabolic process in Control group and regulation of cardiac contraction in Doxo group. The clearance of protein aggregates is mainly done by autophagy. Autophagy occurs at low basal levels in cells to perform homeostatic functions such as protein and organelle turnover (9). Therefore, the presence of aggregates-enriched fractions in Control group may represent a process that is continuously formed under normal conditions and removed by basal autophagy. On the other hand, biological processes like muscle contraction and regulation of heart contraction are representative of the Doxo group. As mentioned before, cardiac muscle contraction is highly dependent of Ca^{2+} signaling and handling (114). In fact, Doxo is known to cause cardiotoxicity by several mechanisms, including disruption of Ca^{2+} homeostasis in cardiomyocytes, and consequently, affecting the conduction system and contractile function of the heart (23). The pro-arrhythmic effect of Doxo was observed

in isolated cardiomyocytes (21). In addition, studies reported that Doxo is capable of induce changes in Ca^{2+} channels present in SR, such as SERCA2a and RyR (134). RyR, SR transmembrane Ca^{2+} channels, are responsible for the release of Ca^{2+} from SR into cytosol, allowing the activation of contractile proteins and heart contraction (134). Disturbed RyR activity/function is implicated in several cardiac diseases, such as HF (135). Interestingly, the type 3 ryanodine receptor (RyR3) was exclusively aggregated on Doxo-treated rabbits, and was the only differentiating protein that made regulation of heart contraction biological process representative of the Doxo group. This result suggests that Doxo may have specific effects on RyR3, compromising its function, and consequently, Ca^{2+} handling and heart contraction. Despite the lack of studies showing an association between Doxo-induced cardiotoxicity and RyR3, evidences report that this drug can bind directly to type 2 ryanodine receptor (RyR2) (136), another isoform of RyR receptors, and alter its activity in cardiomyocytes (134). Moreover, Masumiya *et al.* demonstrated that RyR3 is mainly detected in SA-node and right atrium, whereas RyR2 is expressed in various regions of the heart, suggesting that RyR3 may have a unique role in rhythm generation and conduction. Therefore, RyR3 protein may be a new target for further studies aiming to understand its role in Doxo-induced cardiotoxicity mechanisms. Nevertheless, more studies are needed to confirm this hypothesis. The presence of proteins within aggregates-enriched fractions from Doxo-treated rabbits involved in cardiac conduction may suggest that Doxo promotes loss of function of these proteins, and therefore, changes in conduction system.

Finally, differential protein analysis of aggregates revealed that only one protein, histidine-rich glycoprotein (HRG), is markedly increased in Doxo-treated samples. Histidine-rich glycoprotein is a multidomain plasma glycoprotein composed by 6 distinct domains, consisting of 2 N-terminal domains (N1 and N2), 2 proline-rich regions (PRR1 and PRR2) that flank a histidine-rich region (HRR), and a C-terminal domain. HRG protein binds to a variety of targets, regulating several biological processes, such as immune complex, cell adhesion, coagulation and fibrinolysis (137, 138). This protein has been also implicated in the regulation of angiogenesis, the formation of new blood vessels from pre-existing vessels, in both pro and antiangiogenic manner. The antiangiogenic activity has been attributed to HRR/PRR fragment, which is proteolytically released from the HRG protein (137, 139). Despite the lack of studies showing a relation between HRG or HRR/PRR fragment and Doxo, it is known that this drug can promote changes in angiogenesis, namely have an antiangiogenic effect in tumor cells (140). So, it would be conceivable that treatment with this drug may have a similar effect on cardiomyocytes. Since HRG fragment was markedly increased in Doxo-treated hearts, a hypothesis to explain this finding is that Doxo antiangiogenic effect might be related to proteolysis of HRR/PRR fragment from HRG protein. However, since Doxo-treated rabbits present an early phenotype of Doxo-induced cardiotoxicity,

the aggregation of this fragment could be seen as a cell´s protective response, to prevent antiangiogenic effect of this fragment by eliminating/removing it through aggregation. Therefore, more studies will be necessary to clarify the effect of Doxo on HRG protein and its impact on Doxo-induced cardiotoxicity.

DisGeNET analysis revealed that proteins exclusively aggregated in Doxo-treated hearts were related to cardiovascular diseases, namely interleukin-1 (IL-1) alpha, which was associated with myocardial ischemia and cardiomyopathies. In fact, IL-1 family proteins appear to play a critical role in the pathogenesis of several heart diseases (141). IL-1 alpha is a pro-inflammatory cytokine activated after tissue injury (142). Furthermore, IL-1 seems to participate in Doxo-induced cardiotoxicity, once the use of an IL-1 receptor antagonist led to a reduction in heart damage caused by Doxo (143). However, since our Doxo-treated animals are in an early stage of Doxo-induced cardiotoxicity, the specific effects of Doxo in the aggregation of this protein may be associated with a protective response to prevent deleterious effects of IL-1 in the heart. Beyond that, integrin beta was also identified in DisGeNET network, being related with cardiovascular diseases and HF. It is recognized that integrins display a physiological and cardioprotective function in cardiomyocytes. The β1D integrin was observed to be down-regulated after myocardial infarction (144). Regarding this, the presence of integrin beta in aggregates of Doxo-treated rabbits may be explained by its ineffective protective function in the heart, probably due to changes in its expression and function.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

Protein misfolding and aggregation have been involved in the pathogenesis of several neurodegenerative diseases. We believe that impairment in proteostasis may also contribute to the development of cardiovascular diseases. Based on this proteomic study, we demonstrate that both aging and Doxo treatment can lead to the formation of protein aggregates in the heart tissue. Although levels of protein aggregates-enriched fractions did not differ significantly between Young and Aged groups, neither between Control and Doxo groups, distinct proteins were present in those aggregates shown significant changes in proteostasis in cardiac aging and in cardiotoxic conditions. Among these proteins, the most relevant biological processes observed were related to cardiac structure and function, Ca^{2+} homeostasis and autophagy. Therefore, our results open a new field of research into the role of protein aggregation in aging and in cardiotoxicity conditions. Next step, will be the validation of GeLC-MS/MS results. Also, further research about the proteins differentially expressed in aggregates of each group will be important to understand which underlying mechanisms impact cardiac aging and cardiotoxicity, and subsequently, its potential as novel targets for each of these conditions.

7. REFERENCES

1. Diaz-Villanueva JF, Diaz-Molina R, Garcia-Gonzalez V. Protein Folding and Mechanisms of Proteostasis. International journal of molecular sciences. 2015;16(8):17193-230.
2. Wang X, Su H, Ranek MJ. Protein quality control and degradation in cardiomyocytes. Journal of molecular and cellular cardiology. 2008;45(1):11-27.
3. Sanbe A, Osinska H, Saffitz JE, Glabe CG, Kayed R, Maloyan A, Robbins J. Desmin-related cardiomyopathy in transgenic mice: a cardiac amyloidosis. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(27):10132-6.
4. Hartl FU, Bracher A, Hayer-Hartl M. Molecular chaperones in protein folding and proteostasis. Nature. 2011;475(7356):324-32.
5. Bastos P, Trindade F, Leite-Moreira A, Falcao-Pires I, Ferreira R, Vitorino R. Methodological approaches and insights on protein aggregation in biological systems. Expert review of proteomics. 2017;14(1):55-68.
6. Wang X, Robbins J. Heart failure and protein quality control. Circulation research. 2006;99(12):1315-28.
7. Tannous P, Zhu H, Nemchenko A, Berry JM, Johnstone JL, Shelton JM, Miller FJ, Jr., Rothermel BA, Hill JA. Intracellular protein aggregation is a proximal trigger of cardiomyocyte autophagy. Circulation. 2008;117(24):3070-8.
8. Kanamori H, Takemura G, Maruyama R, Goto K, Tsujimoto A, Ogino A, Li L, Kawamura I, Takeyama T, Kawaguchi T, Nagashima K, Fujiwara T, Fujiwara H, Seishima M, Minatoguchi S. Functional significance and morphological characterization of starvation-induced autophagy in the adult heart. The American journal of pathology. 2009;174(5):1705-14.
9. Lavandero S, Troncoso R, Rothermel BA, Martinet W, Sadoshima J, Hill JA. Cardiovascular autophagy: concepts, controversies, and perspectives. Autophagy. 2013;9(10):1455-66.
10. Spuch C, Ortolano S, Navarro C. New insights in the amyloid-Beta interaction with mitochondria. Journal of aging research. 2012;2012:324968.
11. Chen Q, Liu JB, Horak KM, Zheng H, Kumarapeli AR, Li J, Li F, Gerdes AM, Wawrousek EF, Wang X. Intrasarcoplasmic amyloidosis impairs proteolytic function of proteasomes in cardiomyocytes by compromising substrate uptake. Circulation research. 2005;97(10):1018-26.
12. Cuervo AM, Stefanis L, Fredenburg R, Lansbury PT, Sulzer D. Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy. Science (New York, NY). 2004;305(5688):1292-5.
13. Colombo A, Meroni CA, Cipolla CM, Cardinale D. Managing cardiotoxicity of chemotherapy. Current treatment options in cardiovascular medicine. 2013;15(4):410-24.
14. Townsend N, Nichols M, Scarborough P, Rayner M. Cardiovascular disease in Europe--epidemiological update 2015. European heart journal. 2015;36(40):2696-705.
15. Jugdutt BI. Prevention of heart failure in the elderly: when, where and how to begin? Heart failure reviews. 2012;17(4-5):531-44.
16. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The Hallmarks of Aging. Cell.153(6):1194-217.
17. Squier TC. Oxidative stress and protein aggregation during biological aging. Experimental gerontology. 2001;36(9):1539-50.
18. Ayyadevara S, Mercanti F, Wang X, Mackintosh SG, Tackett AJ, Prayaga SV, Romeo F, Shmookler Reis RJ, Mehta JL. Age- and Hypertension-Associated Protein Aggregates in Mouse Heart Have Similar Proteomic Profiles. Hypertension (Dallas, Tex : 1979). 2016;67(5):1006-13.
19. Taneike M, Yamaguchi O, Nakai A, Hikoso S, Takeda T, Mizote I, Oka T, Tamai T, Oyabu J, Murakawa T, Nishida K, Shimizu T, Hori M, Komuro I, Takuji Shirasawa TS, Mizushima N, Otsu K. Inhibition of autophagy in the heart induces age-related cardiomyopathy. Autophagy. 2010;6(5):600-6.
20. Olivetti G, Melissari M, Capasso JM, Anversa P. Cardiomyopathy of the aging human heart. Myocyte loss and reactive cellular hypertrophy. Circulation research. 1991;68(6):1560-8.
21. Gorelik J, Vodyanoy I, Shevchuk AI, Diakonov IA, Lab MJ, Korchev YE. Esmolol is antiarrhythmic in doxorubicin-induced arrhythmia in cultured cardiomyocytes - determination by novel rapid cardiomyocyte assay. FEBS letters. 2003;548(1-3):74-8.
22. Kumar SN, Konorev EA, Aggarwal D, Kalyanaraman B. Analysis of proteome changes in doxorubicin-treated adult rat cardiomyocyte. Journal of proteomics. 2011;74(5):683-97.
23. Sterba M, Popelova O, Vavrova A, Jirkovsky E, Kovarikova P, Gersl V, Simunek T. Oxidative stress, redox signaling, and metal chelation in anthracycline cardiotoxicity and pharmacological cardioprotection. Antioxidants & redox signaling. 2013;18(8):899-929.

24. da Silva MG, Mattos E, Camacho-Pereira J, Domitrovic T, Galina A, Costa MW, Kurtenbach E. Cardiac systolic dysfunction in doxorubicin-challenged rats is associated with upregulation of MuRF2 and MuRF3 E3 ligases. Experimental and clinical cardiology. 2012;17(3):101-9.
25. Dimitrakis P, Romay-Ogando MI, Timolati F, Suter TM, Zuppinger C. Effects of doxorubicin cancer therapy on autophagy and the ubiquitin-proteasome system in long-term cultured adult rat cardiomyocytes. Cell and tissue research. 2012;350(2):361-72.
26. Toldo S, Goehe RW, Lotrionte M, Mezzaroma E, Sumner ET, Biondi-Zoccai GGL, Seropian IM, Van Tassell BW, Loperfido F, Palazzoni G, Voelkel NF, Abbate A, Gewirtz DA. Comparative Cardiac Toxicity of Anthracyclines In Vitro and In Vivo in the Mouse. PLoS ONE. 2013;8(3):e58421.
27. Ross CA, Poirier MA. What is the role of protein aggregation in neurodegeneration? Nature reviews Molecular cell biology. 2005;6(11):891-8.
28. Vetri V, Canale C, Relini A, Librizzi F, Militello V, Gliozzi A, Leone M. Amyloid fibrils formation and amorphous aggregation in concanavalin A. Biophysical chemistry. 2007;125(1):184-90.
29. Olzmann JA, Li L, Chin LS. Aggresome formation and neurodegenerative diseases: therapeutic implications. Current medicinal chemistry. 2008;15(1):47-60.
30. Huang TH, Yang DS, Fraser PE, Chakrabartty A. Alternate aggregation pathways of the Alzheimer beta-amyloid peptide. An in vitro model of preamyloid. The Journal of biological chemistry. 2000;275(46):36436-40.
31. Merlini G, Bellotti V, Andreola A, Palladini G, Obici L, Casarini S, Perfetti V. Protein Aggregation. Clinical Chemistry and Laboratory Medicine2001. p. 1065.
32. Gonzalez-Lopez E, Gallego-Delgado M, Guzzo-Merello G, de Haro-Del Moral FJ, Cobo-Marcos M, Robles C, Bornstein B, Salas C, Lara-Pezzi E, Alonso-Pulpon L, Garcia-Pavia P. Wild-type transthyretin amyloidosis as a cause of heart failure with preserved ejection fraction. European heart journal. 2015;36(38):2585-94.
33. Terry RD, Masliah E, Salmon DP, Butters N, DeTeresa R, Hill R, Hansen LA, Katzman R. Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. Annals of neurology. 1991;30(4):572-80.
34. Tyedmers J, Mogk A, Bukau B. Cellular strategies for controlling protein aggregation. Nature reviews Molecular cell biology. 2010;11(11):777-88.
35. Dalle-Donne I, Aldini G, Carini M, Colombo R, Rossi R, Milzani A. Protein carbonylation, cellular dysfunction, and disease progression. Journal of cellular and molecular medicine. 2006;10(2):389-406.
36. Stadtman ER, Levine RL. Protein oxidation. Annals of the New York Academy of Sciences. 2000;899:191-208.
37. Tanase M, Urbanska AM, Zolla V, Clement CC, Huang L, Morozova K, Follo C, Goldberg M, Roda B, Reschiglian P, Santambrogio L. Role of Carbonyl Modifications on Aging-Associated Protein Aggregation. Scientific reports. 2016;6:19311.
38. Bulteau AL, Lundberg KC, Humphries KM, Sadek HA, Szweda PA, Friguet B, Szweda LI. Oxidative modification and inactivation of the proteasome during coronary occlusion/reperfusion. The Journal of biological chemistry. 2001;276(32):30057-63.
39. Predmore JM, Wang P, Davis F, Bartolone S, Westfall MV, Dyke DB, Pagani F, Powell SR, Day SM. Ubiquitin proteasome dysfunction in human hypertrophic and dilated cardiomyopathies. Circulation. 2010;121(8):997-1004.
40. Tarone G, Brancaccio M. Keep your heart in shape: molecular chaperone networks for treating heart disease. Cardiovascular research. 2014;102(3):346-61.
41. Koga H, Kaushik S, Cuervo AM. Protein homeostasis and aging: The importance of exquisite quality control. Ageing research reviews. 2011;10(2):205-15.
42. Thiriet M. Pathogenesis of Cardiac Diseases. Diseases of the Cardiac Pump: Springer International Publishing; 2015. p. 1-98.
43. Negro A, Dodge-Kafka K, Kapiloff MS. Signalosomes as Therapeutic Targets. Progress in pediatric cardiology. 2008;25(1):51-6.
44. Willis MS, Patterson C. Hold me tight: Role of the heat shock protein family of chaperones in cardiac disease. Circulation. 2010;122(17):1740-51.
45. Siegers K, Waldmann T, Leroux MR, Grein K, Shevchenko A, Schiebel E, Hartl FU. Compartmentation of protein folding in vivo: sequestration of non-native polypeptide by the chaperonin-GimC system. The EMBO Journal. 1999;18(1):75-84.
46. McClellan AJ, Xia Y, Deutschbauer AM, Davis RW, Gerstein M, Frydman J. Diverse cellular functions of the Hsp90 molecular chaperone uncovered using systems approaches. Cell. 2007;131(1):121-35.

47. Sun Y, MacRae TH. The small heat shock proteins and their role in human disease. The FEBS journal. 2005;272(11):2613-27.
48. Fu HY, Sanada S, Matsuzaki T, Liao Y, Okuda K, Yamato M, Tsuchida S, Araki R, Asano Y, Asanuma H, Asakura M, French BA, Sakata Y, Kitakaze M, Minamino T. Chemical Endoplasmic Reticulum Chaperone Alleviates Doxorubicin-Induced Cardiac Dysfunction. Circulation research. 2016;118(5):798-809.
49. Castro JP, Ott C, Jung T, Grune T, Almeida H. Carbonylation of the cytoskeletal protein actin leads to aggregate formation. Free radical biology & medicine. 2012;53(4):916-25.
50. Pagan J, Seto T, Pagano M, Cittadini A. Role of the ubiquitin proteasome system in the heart. Circulation research. 2013;112(7):1046-58.
51. Willis MS, Patterson C. Into the heart: the emerging role of the ubiquitin-proteasome system. Journal of molecular and cellular cardiology. 2006;41(4):567-79.
52. McLendon PM, Robbins J. Proteotoxicity and Cardiac Dysfunction. Circulation research. 2015;116(11):1863-82.
53. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, Adachi H, Adams CM, Adams PD, Adeli K, Adhihetty PJ, Adler SG, Agam G, Agarwal R, Aghi MK, Agnello M, Agostinis P, Aguilar PV, Aguirre-Ghiso J, Airoldi EM, Ait-Si-Ali S, Akematsu T, Akporiaye ET, Al-Rubeai M, Albaiceta GM, Albanese C, Albani D, Albert ML, Aldudo J, Algul H, Alirezaei M, Alloza I, Almasan A, Almonte-Beceril M, Alnemri ES, Alonso C, Altan-Bonnet N, Altieri DC, Alvarez S, Alvarez-Erviti L, Alves S, Amadoro G, Amano A, Amantini C, Ambrosio S, Amelio I, Amer AO, Amessou M, Amon A, An Z, Anania FA, Andersen SU, Andley UP, Andreadi CK, Andrieu-Abadie N, Anel A, Ann DK, Anoopkumar-Dukie S, Antonioli M, Aoki H, Apostolova N, Aquila S, Aquilano K, Araki K, Arama E, Aranda A, Araya J, Arcaro A, Arias E, Arimoto H, Ariosa AR, Armstrong JL, Arnould T, Arsov I, Asanuma K, Askanas V, Asselin E, Atarashi R, Atherton SS, Atkin JD, Attardi LD, Auberger P, Auburger G, Aurelian L, Autelli R, Avagliano L, Avantaggiati ML, Avrahami L, Awale S, Azad N, Bachetti T, Backer JM, Bae DH, Bae JS, Bae ON, Bae SH, Baehrecke EH, Baek SH, Baghdiguian S, Bagniewska-Zadworna A, Bai H, Bai J, Bai XY, Bailly Y, Balaji KN, Balduini W, Ballabio A, Balzan R, Banerjee R, Banhegyi G, Bao H, Barbeau B, Barrachina MD, Barreiro E, Bartel B, Bartolome A, Bassham DC, Bassi MT, Bast RC, Jr., Basu A, Batista MT, Batoko H, Battino M, Bauckman K, Baumgarner BL, Bayer KU, Beale R, Beaulieu JF, Beck GR, Jr., Becker C, Beckham JD, Bedard PA, Bednarski PJ, Begley TJ, Behl C, Behrends C, Behrens GM, Behrns KE, Bejarano E, Belaid A, Belleudi F, Benard G, Berchem G, Bergamaschi D, Bergami M, Berkhout B, Berliocchi L, Bernard A, Bernard M, Bernassola F, Bertolotti A, Bess AS, Besteiro S, Bettuzzi S, Bhalla S, Bhattacharyya S, Bhutia SK, Biagosch C, Bianchi MW, Biard-Piechaczyk M, Billes V, Bincoletto C, Bingol B, Bird SW, Bitoun M, Bjedov I, Blackstone C, Blanc L, Blanco GA, Blomhoff HK, Boada-Romero E, Bockler S, Boes M, Boesze-Battaglia K, Boise LH, Bolino A, Boman A, Bonaldo P, Bordi M, Bosch J, Botana LM, Botti J, Bou G, Bouche M, Bouchecareilh M, Boucher MJ, Boulton ME, Bouret SG, Boya P, Boyer-Guittaut M, Bozhkov PV, Brady N, Braga VM, Brancolini C, Braus GH, Bravo-San Pedro JM, Brennan LA, Bresnick EH, Brest P, Bridges D, Bringer MA, Brini M, Brito GC, Brodin B, Brookes PS, Brown EJ, Brown K, Broxmeyer HE, Bruhat A, Brum PC, Brumell JH, Brunetti-Pierri N, Bryson-Richardson RJ, Buch S, Buchan AM, Budak H, Bulavin DV, Bultman SJ, Bultynck G, Bumbasirevic V, Burelle Y, Burke RE, Burmeister M, Butikofer P, Caberlotto L, Cadwell K, Cahova M, Cai D, Cai J, Cai Q, Calatayud S, Camougrand N, Campanella M, Campbell GR, Campbell M, Campello S, Candau R, Caniggia I, Cantoni L, Cao L, Caplan AB, Caraglia M, Cardinali C, Cardoso SM, Carew JS, Carleton LA, Carlin CR, Carloni S, Carlsson SR, Carmona-Gutierrez D, Carneiro LA, Carnevali O, Carra S, Carrier A, Carroll B, Casas C, Casas J, Cassinelli G, Castets P, Castro-Obregon S, Cavallini G, Ceccherini I, Cecconi F, Cederbaum AI, Cena V, Cenci S, Cerella C, Cervia D, Cetrullo S, Chaachouay H, Chae HJ, Chagin AS, Chai CY, Chakrabarti G, Chamilos G, Chan EY, Chan MT, Chandra D, Chandra P, Chang CP, Chang RC, Chang TY, Chatham JC, Chatterjee S, Chauhan S, Che Y, Cheetham ME, Cheluvappa R, Chen CJ, Chen G, Chen GC, Chen G, Chen H, Chen JW, Chen JK, Chen M, Chen M, Chen P, Chen Q, Chen Q, Chen SD, Chen S, Chen SS, Chen W, Chen WJ, Chen WQ, Chen W, Chen X, Chen YH, Chen YG, Chen Y, Chen Y, Chen Y, Chen YJ, Chen YQ, Chen Y, Chen Z, Chen Z, Cheng A, Cheng CH, Cheng H, Cheong H, Cherry S, Chesney J, Cheung CH, Chevet E, Chi HC, Chi SG, Chiacchiera F, Chiang HL, Chiarelli R, Chiariello M, Chieppa M, Chin LS, Chiong M, Chiu GN, Cho DH, Cho SG, Cho WC, Cho YY, Cho YS, Choi AM, Choi EJ, Choi EK, Choi J, Choi ME, Choi SI, Chou TF, Chouaib S, Choubey D, Choubey V, Chow KC, Chowdhury K, Chu CT, Chuang TH, Chun T, Chung H, Chung T, Chung YL, Chwae YJ, Cianfanelli V, Ciarcia R, Ciechomska IA, Ciriolo MR, Cirone M, Claerhout S, Clague MJ, Claria J, Clarke PG, Clarke R, Clementi E, Cleyrat C, Cnop M, Coccia EM, Cocco T, Codogno P, Coers J, Cohen EE, Colecchia D, Coletto L, Coll NS, Colucci-Guyon E, Comincini S, Condello M, Cook KL, Coombs GH, Cooper CD, Cooper JM, Coppens I, Corasaniti MT, Corazzari M, Corbalan R, Corcelle-Termeau E, Cordero MD, Corral-Ramos C, Corti O, Cossarizza A,

Costelli P, Costes S, Cotman SL, Coto-Montes A, Cottet S, Couve E, Covey LR, Cowart LA, Cox JS, Coxon FP, Coyne CB, Cragg MS, Craven RJ, Crepaldi T, Crespo JL, Criollo A, Crippa V, Cruz MT, Cuervo AM, Cuezva JM, Cui T, Cutillas PR, Czaja MJ, Czyzyk-Krzeska MF, Dagda RK, Dahmen U, Dai C, Dai W, Dai Y, Dalby KN, Dalla Valle L, Dalmasso G, D'Amelio M, Damme M, Darfeuille-Michaud A, Dargemont C, Darley-Usmar VM, Dasarathy S, Dasgupta B, Dash S, Dass CR, Davey HM, Davids LM, Davila D, Davis RJ, Dawson TM, Dawson VL, Daza P, de Belleroche J, de Figueiredo P, de Figueiredo RC, de la Fuente J, De Martino L, De Matteis A, De Meyer GR, De Milito A, De Santi M, de Souza W, De Tata V, De Zio D, Debnath J, Dechant R, Decuypere JP, Deegan S, Dehay B, Del Bello B, Del Re DP, Delage-Mourroux R, Delbridge LM, Deldicque L, Delorme-Axford E, Deng Y, Dengjel J, Denizot M, Dent P, Der CJ, Deretic V, Derrien B, Deutsch E, Devarenne TP, Devenish RJ, Di Bartolomeo S, Di Daniele N, Di Domenico F, Di Nardo A, Di Paola S, Di Pietro A, Di Renzo L, DiAntonio A, Diaz-Araya G, Diaz-Laviada I, Diaz-Meco MT, Diaz-Nido J, Dickey CA, Dickson RC, Diederich M, Digard P, Dikic I, Dinesh-Kumar SP, Ding C, Ding WX, Ding Z, Dini L, Distler JH, Diwan A, Djavaheri-Mergny M, Dmytruk K, Dobson RC, Doetsch V, Dokladny K, Dokudovskaya S, Donadelli M, Dong XC, Dong X, Dong Z, Donohue TM, Jr., Doran KS, D'Orazi G, Dorn GW, 2nd, Dosenko V, Dridi S, Drucker L, Du J, Du LL, Du L, du Toit A, Dua P, Duan L, Duann P, Dubey VK, Duchen MR, Duchosal MA, Duez H, Dugail I, Dumit VI, Duncan MC, Dunlop EA, Dunn WA, Jr., Dupont N, Dupuis L, Duran RV, Durcan TM, Duvezin-Caubet S, Duvvuri U, Eapen V, Ebrahimi-Fakhari D, Echard A, Eckhart L, Edelstein CL, Edinger AL, Eichinger L, Eisenberg T, Eisenberg-Lerner A, Eissa NT, El-Deiry WS, El-Khoury V, Elazar Z, Eldar-Finkelman H, Elliott CJ, Emanuele E, Emmenegger U, Engedal N, Engelbrecht AM, Engelender S, Enserink JM, Erdmann R, Erenpreisa J, Eri R, Eriksen JL, Erman A, Escalante R, Eskelinen EL, Espert L, Esteban-Martinez L, Evans TJ, Fabri M, Fabrias G, Fabrizi C, Facchiano A, Faergeman NJ, Faggioni A, Fairlie WD, Fan C, Fan D, Fan J, Fang S, Fanto M, Fanzani A, Farkas T, Faure M, Favier FB, Fearnhead H, Federici M, Fei E, Felizardo TC, Feng H, Feng Y, Feng Y, Ferguson TA, Fernandez AF, Fernandez-Barrena MG, Fernandez-Checa JC, Fernandez-Lopez A, Fernandez-Zapico ME, Feron O, Ferraro E, Ferreira-Halder CV, Fesus L, Feuer R, Fiesel FC, Filippi-Chiela EC, Filomeni G, Fimia GM, Fingert JH, Finkbeiner S, Finkel T, Fiorito F, Fisher PB, Flajolet M, Flamigni F, Florey O, Florio S, Floto RA, Folini M, Follo C, Fon EA, Fornai F, Fortunato F, Fraldi A, Franco R, Francois A, Francois A, Frankel LB, Fraser ID, Frey N, Freyssenet DG, Frezza C, Friedman SL, Frigo DE, Fu D, Fuentes JM, Fueyo J, Fujitani Y, Fujiwara Y, Fujiya M, Fukuda M, Fulda S, Fusco C, Gabryel B, Gaestel M, Gailly P, Gajewska M, Galadari S, Galili G, Galindo I, Galindo MF, Galliciotti G, Galluzzi L, Galluzzi L, Galy V, Gammoh N, Gandy S, Ganesan AK, Ganesan S, Ganley IG, Gannage M, Gao FB, Gao F, Gao JX, Garcia Nannig L, Garcia Vescovi E, Garcia-Macia M, Garcia-Ruiz C, Garg AD, Garg PK, Gargini R, Gassen NC, Gatica D, Gatti E, Gavard J, Gavathiotis E, Ge L, Ge P, Ge S, Gean PW, Gelmetti V, Genazzani AA, Geng J, Genschik P, Gerner L, Gestwicki JE, Gewirtz DA, Ghavami S, Ghigo E, Ghosh D, Giammarioli AM, Giampieri F, Giampietri C, Giatromanolaki A, Gibbings DJ, Gibellini L, Gibson SB, Ginet V, Giordano A, Giorgini F, Giovannetti E, Girardin SE, Gispert S, Giuliano S, Gladson CL, Glavic A, Gleave M, Godefroy N, Gogal RM, Jr., Gokulan K, Goldman GH, Goletti D, Goligorsky MS, Gomes AV, Gomes LC, Gomez H, Gomez-Manzano C, Gomez-Sanchez R, Goncalves DA, Goncu E, Gong Q, Gongora C, Gonzalez CB, Gonzalez-Alegre P, Gonzalez-Cabo P, Gonzalez-Polo RA, Goping IS, Gorbea C, Gorbunov NV, Goring DR, Gorman AM, Gorski SM, Goruppi S, Goto-Yamada S, Gotor C, Gottlieb RA, Gozes I, Gozuacik D, Graba Y, Graef M, Granato GE, Grant GD, Grant S, Gravina GL, Green DR, Greenhough A, Greenwood MT, Grimaldi B, Gros F, Grose C, Groulx JF, Gruber F, Grumati P, Grune T, Guan JL, Guan KL, Guerra B, Guillen C, Gulshan K, Gunst J, Guo C, Guo L, Guo M, Guo W, Guo XG, Gust AA, Gustafsson AB, Gutierrez E, Gutierrez MG, Gwak HS, Haas A, Haber JE, Hadano S, Hagedorn M, Hahn DR, Halayko AJ, Hamacher-Brady A, Hamada K, Hamai A, Hamann A, Hamasaki M, Hamer I, Hamid Q, Hammond EM, Han F, Han W, Handa JT, Hanover JA, Hansen M, Harada M, Harhaji-Trajkovic L, Harper JW, Harrath AH, Harris AL, Harris J, Hasler U, Hasselblatt P, Hasui K, Hawley RG, Hawley TS, He C, He CY, He F, He G, He RR, He XH, He YW, He YY, Heath JK, Hebert MJ, Heinzen RA, Helgason GV, Hensel M, Henske EP, Her C, Herman PK, Hernandez A, Hernandez C, Hernandez-Tiedra S, Hetz C, Hiesinger PR, Higaki K, Hilfiker S, Hill BG, Hill JA, Hill WD, Hino K, Hofius D, Hofman P, Hoglinger GU, Hohfeld J, Holz MK, Hong Y, Hood DA, Hoozemans JJ, Hoppe T, Hsu C, Hsu CY, Hsu LC, Hu D, Hu G, Hu HM, Hu H, Hu MC, Hu YC, Hu ZW, Hua F, Hua Y, Huang C, Huang HL, Huang KH, Huang KY, Huang S, Huang S, Huang WP, Huang YR, Huang Y, Huang Y, Huber TB, Huebbe P, Huh WK, Hulmi JJ, Hur GM, Hurley JH, Husak Z, Hussain SN, Hussain S, Hwang JJ, Hwang S, Hwang TI, Ichihara A, Imai Y, Imbriano C, Inomata M, Into T, Iovane V, Iovanna JL, Iozzo RV, Ip NY, Irazoqui JE, Iribarren P, Isaka Y, Isakovic AJ, Ischiropoulos H, Isenberg JS, Ishaq M, Ishida H, Ishii I, Ishmael JE, Isidoro C, Isobe K, Isono E, Issazadeh-Navikas S, Itahana K, Itakura E, Ivanov AI, Iyer AK, Izquierdo JM, Izumi Y, Izzo V, Jaattela M, Jaber N, Jackson DJ, Jackson WT, Jacob TG, Jacques TS, Jagannath C, Jain A, Jana NR, Jang BK, Jani A, Janji B, Jannig PR, Jansson PJ,

Jean S, Jendrach M, Jeon JH, Jessen N, Jeung EB, Jia K, Jia L, Jiang H, Jiang H, Jiang L, Jiang T, Jiang X, Jiang X, Jiang X, Jiang Y, Jiang Y, Jimenez A, Jin C, Jin H, Jin L, Jin M, Jin S, Jinwal UK, Jo EK, Johansen T, Johnson DE, Johnson GV, Johnson JD, Jonasch E, Jones C, Joosten LA, Jordan J, Joseph AM, Joseph B, Joubert AM, Ju D, Ju J, Juan HF, Juenemann K, Juhasz G, Jung HS, Jung JU, Jung YK, Jungbluth H, Justice MJ, Jutten B, Kaakoush NO, Kaarniranta K, Kaasik A, Kabuta T, Kaeffer B, Kagedal K, Kahana A, Kajimura S, Kakhlon O, Kalia M, Kalvakolanu DV, Kamada Y, Kambas K, Kaminskyy VO, Kampinga HH, Kandouz M, Kang C, Kang R, Kang TC, Kanki T, Kanneganti TD, Kanno H, Kanthasamy AG, Kantorow M, Kaparakis-Liaskos M, Kapuy O, Karantza V, Karim MR, Karmakar P, Kaser A, Kaushik S, Kawula T, Kaynar AM, Ke PY, Ke ZJ, Kehrl JH, Keller KE, Kemper JK, Kenworthy AK, Kepp O, Kern A, Kesari S, Kessel D, Ketteler R, Kettelhut Ido C, Khambu B, Khan MM, Khandelwal VK, Khare S, Kiang JG, Kiger AA, Kihara A, Kim AL, Kim CH, Kim DR, Kim DH, Kim EK, Kim HY, Kim HR, Kim JS, Kim JH, Kim JC, Kim JH, Kim KW, Kim MD, Kim MM, Kim PK, Kim SW, Kim SY, Kim YS, Kim Y, Kimchi A, Kimmelman AC, Kimura T, King JS, Kirkegaard K, Kirkin V, Kirshenbaum LA, Kishi S, Kitajima Y, Kitamoto K, Kitaoka Y, Kitazato K, Kley RA, Klimecki WT, Klinkenberg M, Klucken J, Knaevelsrud H, Knecht E, Knuppertz L, Ko JL, Kobayashi S, Koch JC, Koechlin-Ramonatxo C, Koenig U, Koh YH, Kohler K, Kohlwein SD, Koike M, Komatsu M, Kominami E, Kong D, Kong HJ, Konstantakou EG, Kopp BT, Korcsmaros T, Korhonen L, Korolchuk VI, Koshkina NV, Kou Y, Koukourakis MI, Koumenis C, Kovacs AL, Kovacs T, Kovacs WJ, Koya D, Kraft C, Krainc D, Kramer H, Kravic-Stevovic T, Krek W, Kretz-Remy C, Krick R, Krishnamurthy M, Kriston-Vizi J, Kroemer G, Kruer MC, Kruger R, Ktistakis NT, Kuchitsu K, Kuhn C, Kumar AP, Kumar A, Kumar A, Kumar D, Kumar D, Kumar R, Kumar S, Kundu M, Kung HJ, Kuno A, Kuo SH, Kuret J, Kurz T, Kwok T, Kwon TK, Kwon YT, Kyrmizi I, La Spada AR, Lafont F, Lahm T, Lakkaraju A, Lam T, Lamark T, Lancel S, Landowski TH, Lane DJ, Lane JD, Lanzi C, Lapaquette P, Lapierre LR, Laporte J, Laukkarinen J, Laurie GW, Lavandero S, Lavie L, LaVoie MJ, Law BY, Law HK, Law KB, Layfield R, Lazo PA, Le Cam L, Le Roch KG, Le Stunff H, Leardkamolkarn V, Lecuit M, Lee BH, Lee CH, Lee EF, Lee GM, Lee HJ, Lee H, Lee JK, Lee J, Lee JH, Lee JH, Lee M, Lee MS, Lee PJ, Lee SW, Lee SJ, Lee SJ, Lee SY, Lee SH, Lee SS, Lee SJ, Lee S, Lee YR, Lee YJ, Lee YH, Leeuwenburgh C, Lefort S, Legouis R, Lei J, Lei QY, Leib DA, Leibowitz G, Lekli I, Lemaire SD, Lemasters JJ, Lemberg MK, Lemoine A, Leng S, Lenz G, Lenzi P, Lerman LO, Lettieri Barbato D, Leu JI, Leung HY, Levine B, Lewis PA, Lezoualc'h F, Li C, Li F, Li FJ, Li J, Li K, Li L, Li M, Li M, Li Q, Li R, Li S, Li W, Li W, Li X, Li Y, Lian J, Liang C, Liang Q, Liao Y, Liberal J, Liberski PP, Lie P, Lieberman AP, Lim HJ, Lim KL, Lim K, Lima RT, Lin CS, Lin CF, Lin F, Lin F, Lin FC, Lin K, Lin KH, Lin PH, Lin T, Lin WW, Lin YS, Lin Y, Linden R, Lindholm D, Lindqvist LM, Lingor P, Linkermann A, Liotta LA, Lipinski MM, Lira VA, Lisanti MP, Liton PB, Liu B, Liu C, Liu CF, Liu F, Liu HJ, Liu J, Liu JJ, Liu JL, Liu K, Liu L, Liu L, Liu Q, Liu RY, Liu S, Liu S, Liu W, Liu XD, Liu X, Liu XH, Liu X, Liu X, Liu X, Liu Y, Liu Y, Liu Z, Liu Z, Liuzzi JP, Lizard G, Ljujic M, Lodhi IJ, Logue SE, Lokeshwar BL, Long YC, Lonial S, Loos B, Lopez-Otin C, Lopez-Vicario C, Lorente M, Lorenzi PL, Lorincz P, Los M, Lotze MT, Lovat PE, Lu B, Lu B, Lu J, Lu Q, Lu SM, Lu S, Lu Y, Luciano F, Luckhart S, Lucocq JM, Ludovico P, Lugea A, Lukacs NW, Lum JJ, Lund AH, Luo H, Luo J, Luo S, Luparello C, Lyons T, Ma J, Ma Y, Ma Y, Ma Z, Machado J, Machado-Santelli GM, Macian F, MacIntosh GC, MacKeigan JP, Macleod KF, MacMicking JD, MacMillan-Crow LA, Madeo F, Madesh M, Madrigal-Matute J, Maeda A, Maeda T, Maegawa G, Maellaro E, Maes H, Magarinos M, Maiese K, Maiti TK, Maiuri L, Maiuri MC, Maki CG, Malli R, Malorni W, Maloyan A, Mami-Chouaib F, Man N, Mancias JD, Mandelkow EM, Mandell MA, Manfredi AA, Manie SN, Manzoni C, Mao K, Mao Z, Mao ZW, Marambaud P, Marconi AM, Marelja Z, Marfe G, Margeta M, Margittai E, Mari M, Mariani FV, Marin C, Marinelli S, Marino G, Markovic I, Marquez R, Martelli AM, Martens S, Martin KR, Martin SJ, Martin S, Martin-Acebes MA, Martin-Sanz P, Martinand-Mari C, Martinet W, Martinez J, Martinez-Lopez N, Martinez-Outschoorn U, Martinez-Velazquez M, Martinez-Vicente M, Martins WK, Mashima H, Mastrianni JA, Matarese G, Matarrese P, Mateo R, Matoba S, Matsumoto N, Matsushita T, Matsuura A, Matsuzawa T, Mattson MP, Matus S, Maugeri N, Mauvezin C, Mayer A, Maysinger D, Mazzolini GD, McBrayer MK, McCall K, McCormick C, McInerney GM, McIver SC, McKenna S, McMahon JJ, McNeish IA, Mechta-Grigoriou F, Medema JP, Medina DL, Megyeri K, Mehrpour M, Mehta JL, Mei Y, Meier UC, Meijer AJ, Melendez A, Melino G, Melino S, de Melo EJ, Mena MA, Meneghini MD, Menendez JA, Menezes R, Meng L, Meng LH, Meng S, Menghini R, Menko AS, Menna-Barreto RF, Menon MB, Meraz-Rios MA, Merla G, Merlini L, Merlot AM, Meryk A, Meschini S, Meyer JN, Mi MT, Miao CY, Micale L, Michaeli S, Michiels C, Migliaccio AR, Mihailidou AS, Mijaljica D, Mikoshiba K, Milan E, Miller-Fleming L, Mills GB, Mills IG, Minakaki G, Minassian BA, Ming XF, Minibayeva F, Minina EA, Mintern JD, Minucci S, Miranda-Vizuete A, Mitchell CH, Miyamoto S, Miyazawa K, Mizushima N, Mnich K, Mograbi B, Mohseni S, Moita LF, Molinari M, Molinari M, Moller AB, Mollereau B, Mollinedo F, Mongillo M, Monick MM, Montagnaro S, Montell C, Moore DJ, Moore MN, Mora-Rodriguez R, Moreira PI, Morel E,

Morelli MB, Moreno S, Morgan MJ, Moris A, Moriyasu Y, Morrison JL, Morrison LA, Morselli E, Moscat J, Moseley PL, Mostowy S, Motori E, Mottet D, Mottram JC, Moussa CE, Mpakou VE, Mukhtar H, Mulcahy Levy JM, Muller S, Munoz-Moreno R, Munoz-Pinedo C, Munz C, Murphy ME, Murray JT, Murthy A, Mysorekar IU, Nabi IR, Nabissi M, Nader GA, Nagahara Y, Nagai Y, Nagata K, Nagelkerke A, Nagy P, Naidu SR, Nair S, Nakano H, Nakatogawa H, Nanjundan M, Napolitano G, Naqvi NI, Nardacci R, Narendra DP, Narita M, Nascimbeni AC, Natarajan R, Navegantes LC, Nawrocki ST, Nazarko TY, Nazarko VY, Neill T, Neri LM, Netea MG, Netea-Maier RT, Neves BM, Ney PA, Nezis IP, Nguyen HT, Nguyen HP, Nicot AS, Nilsen H, Nilsson P, Nishimura M, Nishino I, Niso-Santano M, Niu H, Nixon RA, Njar VC, Noda T, Noegel AA, Nolte EM, Norberg E, Norga KK, Noureini SK, Notomi S, Notterpek L, Nowikovsky K, Nukina N, Nurnberger T, O'Donnell VB, O'Donovan T, O'Dwyer PJ, Oehme I, Oeste CL, Ogawa M, Ogretmen B, Ogura Y, Oh YJ, Ohmuraya M, Ohshima T, Ojha R, Okamoto K, Okazaki T, Oliver FJ, Ollinger K, Olsson S, Orban DP, Ordonez P, Orhon I, Orosz L, O'Rourke EJ, Orozco H, Ortega AL, Ortona E, Osellame LD, Oshima J, Oshima S, Osiewacz HD, Otomo T, Otsu K, Ou JH, Outeiro TF, Ouyang DY, Ouyang H, Overholtzer M, Ozbun MA, Ozdinler PH, Ozpolat B, Pacelli C, Paganetti P, Page G, Pages G, Pagnini U, Pajak B, Pak SC, Pakos-Zebrucka K, Pakpour N, Palkova Z, Palladino F, Pallauf K, Pallet N, Palmieri M, Paludan SR, Palumbo C, Palumbo S, Pampliega O, Pan H, Pan W, Panaretakis T, Pandey A, Pantazopoulou A, Papackova Z, Papademetrio DL, Papassideri I, Papini A, Parajuli N, Pardo J, Parekh VV, Parenti G, Park JI, Park J, Park OK, Parker R, Parlato R, Parys JB, Parzych KR, Pasquet JM, Pasquier B, Pasumarthi KB, Patschan D, Patterson C, Pattingre S, Pattison S, Pause A, Pavenstadt H, Pavone F, Pedrozo Z, Pena FJ, Penalva MA, Pende M, Peng J, Penna F, Penninger JM, Pensalfini A, Pepe S, Pereira GJ, Pereira PC, Perez-de la Cruz V, Perez-Perez ME, Perez-Rodriguez D, Perez-Sala D, Perier C, Perl A, Perlmutter DH, Perrotta I, Pervaiz S, Pesonen M, Pessin JE, Peters GJ, Petersen M, Petrache I, Petrof BJ, Petrovski G, Phang JM, Piacentini M, Pierdominici M, Pierre P, Pierrefite-Carle V, Pietrocola F, Pimentel-Muinos FX, Pinar M, Pineda B, Pinkas-Kramarski R, Pinti M, Pinton P, Piperdi B, Piret JM, Platanias LC, Platta HW, Plowey ED, Poggeler S, Poirot M, Polcic P, Poletti A, Poon AH, Popelka H, Popova B, Poprawa I, Poulose SM, Poulton J, Powers SK, Powers T, Pozuelo-Rubio M, Prak K, Prange R, Prescott M, Priault M, Prince S, Proia RL, Proikas-Cezanne T, Prokisch H, Promponas VJ, Przyklenk K, Puertollano R, Pugazhenthi S, Puglielli L, Pujol A, Puyal J, Pyeon D, Qi X, Qian WB, Qin ZH, Qiu Y, Qu Z, Quadrilatero J, Quinn F, Raben N, Rabinowich H, Radogna F, Ragusa MJ, Rahmani M, Raina K, Ramanadham S, Ramesh R, Rami A, Randall-Demllo S, Randow F, Rao H, Rao VA, Rasmussen BB, Rasse TM, Ratovitski EA, Rautou PE, Ray SK, Razani B, Reed BH, Reggiori F, Rehm M, Reichert AS, Rein T, Reiner DJ, Reits E, Ren J, Ren X, Renna M, Reusch JE, Revuelta JL, Reyes L, Rezaie AR, Richards RI, Richardson DR, Richetta C, Riehle MA, Rihn BH, Rikihisa Y, Riley BE, Rimbach G, Rippo MR, Ritis K, Rizzi F, Rizzo E, Roach PJ, Robbins J, Roberge M, Roca G, Roccheri MC, Rocha S, Rodrigues CM, Rodriguez CI, de Cordoba SR, Rodriguez-Muela N, Roelofs J, Rogov VV, Rohn TT, Rohrer B, Romanelli D, Romani L, Romano PS, Roncero MI, Rosa JL, Rosello A, Rosen KV, Rosenstiel P, Rost-Roszkowska M, Roth KA, Roue G, Rouis M, Rouschop KM, Ruan DT, Ruano D, Rubinsztein DC, Rucker EB, 3rd, Rudich A, Rudolf E, Rudolf R, Ruegg MA, Ruiz-Roldan C, Ruparelia AA, Rusmini P, Russ DW, Russo GL, Russo G, Russo R, Rusten TE, Ryabovol V, Ryan KM, Ryter SW, Sabatini DM, Sacher M, Sachse C, Sack MN, Sadoshima J, Saftig P, Sagi-Eisenberg R, Sahni S, Saikumar P, Saito T, Saitoh T, Sakakura K, Sakoh-Nakatogawa M, Sakuraba Y, Salazar-Roa M, Salomoni P, Saluja AK, Salvaterra PM, Salvioli R, Samali A, Sanchez AM, Sanchez-Alcazar JA, Sanchez-Prieto R, Sandri M, Sanjuan MA, Santaguida S, Santambrogio L, Santoni G, Dos Santos CN, Saran S, Sardiello M, Sargent G, Sarkar P, Sarkar S, Sarrias MR, Sarwal MM, Sasakawa C, Sasaki M, Sass M, Sato K, Sato M, Satriano J, Savaraj N, Saveljeva S, Schaefer L, Schaible UE, Scharl M, Schatzl HM, Schekman R, Scheper W, Schiavi A, Schipper HM, Schmeisser H, Schmidt J, Schmitz I, Schneider BE, Schneider EM, Schneider JL, Schon EA, Schonenberger MJ, Schonthal AH, Schorderet DF, Schroder B, Schuck S, Schulze RJ, Schwarten M, Schwarz TL, Sciarretta S, Scotto K, Scovassi AI, Screaton RA, Screen M, Seca H, Sedej S, Segatori L, Segev N, Seglen PO, Segui-Simarro JM, Segura-Aguilar J, Seki E, Sell C, Seiliez I, Semenkovich CF, Semenza GL, Sen U, Serra AL, Serrano-Puebla A, Sesaki H, Setoguchi T, Settembre C, Shacka JJ, Shajahan-Haq AN, Shapiro IM, Sharma S, She H, Shen CK, Shen CC, Shen HM, Shen S, Shen W, Sheng R, Sheng X, Sheng ZH, Shepherd TG, Shi J, Shi Q, Shi Q, Shi Y, Shibutani S, Shibuya K, Shidoji Y, Shieh JJ, Shih CM, Shimada Y, Shimizu S, Shin DW, Shinohara ML, Shintani M, Shintani T, Shioi T, Shirabe K, Shiri-Sverdlov R, Shirihai O, Shore GC, Shu CW, Shukla D, Sibirny AA, Sica V, Sigurdson CJ, Sigurdsson EM, Sijwali PS, Sikorska B, Silveira WA, Silvente-Poirot S, Silverman GA, Simak J, Simmet T, Simon AK, Simon HU, Simone C, Simons M, Simonsen A, Singh R, Singh SV, Singh SK, Sinha D, Sinha S, Sinicrope FA, Sirko A, Sirohi K, Sishi BJ, Sittler A, Siu PM, Sivridis E, Skwarska A, Slack R, Slaninova I, Slavov N, Smaili SS, Smalley KS, Smith DR, Soenen SJ, Soleimanpour SA, Solhaug A, Somasundaram K, Son JH, Sonawane A, Song C, Song F, Song HK, Song JX, Song W, Soo KY, Sood AK, Soong TW,

Soontornniyomkij V, Sorice M, Sotgia F, Soto-Pantoja DR, Sotthibundhu A, Sousa MJ, Spaink HP, Span PN, Spang A, Sparks JD, Speck PG, Spector SA, Spies CD, Springer W, Clair DS, Stacchiotti A, Staels B, Stang MT, Starczynowski DT, Starokadomskyy P, Steegborn C, Steele JW, Stefanis L, Steffan J, Stellrecht CM, Stenmark H, Stepkowski TM, Stern ST, Stevens C, Stockwell BR, Stoka V, Storchova Z, Stork B, Stratoulias V, Stravopodis DJ, Strnad P, Strohecker AM, Strom AL, Stromhaug P, Stulik J, Su YX, Su Z, Subauste CS, Subramaniam S, Sue CM, Suh SW, Sui X, Sukseree S, Sulzer D, Sun FL, Sun J, Sun J, Sun SY, Sun Y, Sun Y, Sun Y, Sundaramoorthy V, Sung J, Suzuki H, Suzuki K, Suzuki N, Suzuki T, Suzuki YJ, Swanson MS, Swanton C, Sward K, Swarup G, Sweeney ST, Sylvester PW, Szatmari Z, Szegezdi E, Szlosarek PW, Taegtmeyer H, Tafani M, Taillebourg E, Tait SW, Takacs-Vellai K, Takahashi Y, Takats S, Takemura G, Takigawa N, Talbot NJ, Tamagno E, Tamburini J, Tan CP, Tan L, Tan ML, Tan M, Tan YJ, Tanaka K, Tanaka M, Tang D, Tang D, Tang G, Tanida I, Tanji K, Tannous BA, Tapia JA, Tasset-Cuevas I, Tatar M, Tavassoly I, Tavernarakis N, Taylor A, Taylor GS, Taylor GA, Taylor JP, Taylor MJ, Tchetina EV, Tee AR, Teixeira-Clerc F, Telang S, Tencomnao T, Teng BB, Teng RJ, Terro F, Tettamanti G, Theiss AL, Theron AE, Thomas KJ, Thome MP, Thomes PG, Thorburn A, Thorner J, Thum T, Thumm M, Thurston TL, Tian L, Till A, Ting JP, Titorenko VI, Toker L, Toldo S, Tooze SA, Topisirovic I, Torgersen ML, Torosantucci L, Torriglia A, Torrisi MR, Tournier C, Towns R, Trajkovic V, Travassos LH, Triola G, Tripathi DN, Trisciuoglio D, Troncoso R, Trougakos IP, Truttmann AC, Tsai KJ, Tschan MP, Tseng YH, Tsukuba T, Tsung A, Tsvetkov AS, Tu S, Tuan HY, Tucci M, Tumbarello DA, Turk B, Turk V, Turner RF, Tveita AA, Tyagi SC, Ubukata M, Uchiyama Y, Udelnow A, Ueno T, Umekawa M, Umemiya-Shirafuji R, Underwood BR, Ungermann C, Ureshino RP, Ushioda R, Uversky VN, Uzcategui NL, Vaccari T, Vaccaro MI, Vachova L, Vakifahmetoglu-Norberg H, Valdor R, Valente EM, Vallette F, Valverde AM, Van den Berghe G, Van Den Bosch L, van den Brink GR, van der Goot FG, van der Klei IJ, van der Laan LJ, van Doorn WG, van Egmond M, van Golen KL, Van Kaer L, van Lookeren Campagne M, Vandenabeele P, Vandenberghe W, Vanhorebeek I, Varela-Nieto I, Vasconcelos MH, Vasko R, Vavvas DG, Vega-Naredo I, Velasco G, Velentzas AD, Velentzas PD, Vellai T, Vellenga E, Vendelbo MH, Venkatachalam K, Ventura N, Ventura S, Veras PS, Verdier M, Vertessy BG, Viale A, Vidal M, Vieira HL, Vierstra RD, Vigneswaran N, Vij N, Vila M, Villar M, Villar VH, Villarroya J, Vindis C, Viola G, Viscomi MT, Vitale G, Vogl DT, Voitsekhovskaja OV, von Haefen C, von Schwarzenberg K, Voth DE, Vouret-Craviari V, Vuori K, Vyas JM, Waeber C, Walker CL, Walker MJ, Walter J, Wan L, Wan X, Wang B, Wang C, Wang CY, Wang C, Wang C, Wang C, Wang D, Wang F, Wang F, Wang G, Wang HJ, Wang H, Wang HG, Wang H, Wang HD, Wang J, Wang J, Wang M, Wang MQ, Wang PY, Wang P, Wang RC, Wang S, Wang TF, Wang X, Wang XJ, Wang XW, Wang X, Wang X, Wang Y, Wang Y, Wang Y, Wang YJ, Wang Y, Wang Y, Wang YT, Wang Y, Wang ZN, Wappner P, Ward C, Ward DM, Warnes G, Watada H, Watanabe Y, Watase K, Weaver TE, Weekes CD, Wei J, Weide T, Weihl CC, Weindl G, Weis SN, Wen L, Wen X, Wen Y, Westermann B, Weyand CM, White AR, White E, Whitton JL, Whitworth AJ, Wiels J, Wild F, Wildenberg ME, Wileman T, Wilkinson DS, Wilkinson S, Willbold D, Williams C, Williams K, Williamson PR, Winklhofer KF, Witkin SS, Wohlgemuth SE, Wollert T, Wolvetang EJ, Wong E, Wong GW, Wong RW, Wong VK, Woodcock EA, Wright KL, Wu C, Wu D, Wu GS, Wu J, Wu J, Wu M, Wu M, Wu S, Wu WK, Wu Y, Wu Z, Xavier CP, Xavier RJ, Xia GX, Xia T, Xia W, Xia Y, Xiao H, Xiao J, Xiao S, Xiao W, Xie CM, Xie Z, Xie Z, Xilouri M, Xiong Y, Xu C, Xu C, Xu F, Xu H, Xu H, Xu J, Xu J, Xu J, Xu L, Xu X, Xu Y, Xu Y, Xu ZX, Xu Z, Xue Y, Yamada T, Yamamoto A, Yamanaka K, Yamashina S, Yamashiro S, Yan B, Yan B, Yan X, Yan Z, Yanagi Y, Yang DS, Yang JM, Yang L, Yang M, Yang PM, Yang P, Yang Q, Yang W, Yang WY, Yang X, Yang Y, Yang Y, Yang Z, Yang Z, Yao MC, Yao PJ, Yao X, Yao Z, Yao Z, Yasui LS, Ye M, Yedvobnick B, Yeganeh B, Yeh ES, Yeyati PL, Yi F, Yi L, Yin XM, Yip CK, Yoo YM, Yoo YH, Yoon SY, Yoshida K, Yoshimori T, Young KH, Yu H, Yu JJ, Yu JT, Yu J, Yu L, Yu WH, Yu XF, Yu Z, Yuan J, Yuan ZM, Yue BY, Yue J, Yue Z, Zacks DN, Zacksenhaus E, Zaffaroni N, Zaglia T, Zakeri Z, Zecchini V, Zeng J, Zeng M, Zeng Q, Zervos AS, Zhang DD, Zhang F, Zhang G, Zhang GC, Zhang H, Zhang H, Zhang H, Zhang H, Zhang J, Zhang J, Zhang J, Zhang J, Zhang JP, Zhang L, Zhang L, Zhang L, Zhang L, Zhang MY, Zhang X, Zhang XD, Zhang Y, Zhang Y, Zhang Y, Zhang Y, Zhang Y, Zhao M, Zhao WL, Zhao X, Zhao YG, Zhao Y, Zhao Y, Zhao YX, Zhao Z, Zhao ZJ, Zheng D, Zheng XL, Zheng X, Zhivotovsky B, Zhong Q, Zhou GZ, Zhou G, Zhou H, Zhou SF, Zhou XJ, Zhu H, Zhu H, Zhu WG, Zhu W, Zhu XF, Zhu Y, Zhuang SM, Zhuang X, Ziparo E, Zois CE, Zoladek T, Zong WX, Zorzano A, Zughaier SM. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). Autophagy. 2016;12(1):1-222.

54. Leon LJ, Gustafsson AB. Staying young at heart: autophagy and adaptation to cardiac aging. Journal of molecular and cellular cardiology. 2016;95:78-85.

55. Yang YP, Liang ZQ, Gu ZL, Qin ZH. Molecular mechanism and regulation of autophagy. Acta pharmacologica Sinica. 2005;26(12):1421-34.

56. Bjorkoy G, Lamark T, Brech A, Outzen H, Perander M, Overvatn A, Stenmark H, Johansen T. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. The Journal of cell biology. 2005;171(4):603-14.
57. Reggiori F, Komatsu M, Finley K, Simonsen A. Autophagy: more than a nonselective pathway. International journal of cell biology. 2012;2012:219625.
58. Martin-Fernandez B, Gredilla R. Mitochondria and oxidative stress in heart aging. Age (Dordrecht, Netherlands). 2016;38(4):225-38.
59. Marzetti E, Csiszar A, Dutta D, Balagopal G, Calvani R, Leeuwenburgh C. Role of mitochondrial dysfunction and altered autophagy in cardiovascular aging and disease: from mechanisms to therapeutics. American journal of physiology Heart and circulatory physiology. 2013;305(4):H459-76.
60. Hoshino A, Mita Y, Okawa Y, Ariyoshi M, Iwai-Kanai E, Ueyama T, Ikeda K, Ogata T, Matoba S. Cytosolic p53 inhibits Parkin-mediated mitophagy and promotes mitochondrial dysfunction in the mouse heart. Nature communications. 2013;4:2308.
61. Yamaguchi O, Taneike M, Otsu K. Cooperation between proteolytic systems in cardiomyocyte recycling. Cardiovascular research. 2012;96(1):46-52.
62. Galvez AS, Diwan A, Odley AM, Hahn HS, Osinska H, Melendez JG, Robbins J, Lynch RA, Marreez Y, Dorn GW, 2nd. Cardiomyocyte degeneration with calpain deficiency reveals a critical role in protein homeostasis. Circulation research. 2007;100(7):1071-8.
63. Labbadia J, Morimoto RI. The biology of proteostasis in aging and disease. Annual review of biochemistry. 2015;84:435-64.
64. Locke M, Tanguay RM. Diminished heat shock response in the aged myocardium. Cell stress & chaperones. 1996;1(4):251-60.
65. Starnes JW, Choilawala AM, Taylor RP, Nelson MJ, Delp MD. Myocardial heat shock protein 70 expression in young and old rats after identical exercise programs. The journals of gerontology Series A, Biological sciences and medical sciences. 2005;60(8):963-9.
66. Carrard G, Bulteau AL, Petropoulos I, Friguet B. Impairment of proteasome structure and function in aging. The international journal of biochemistry & cell biology. 2002;34(11):1461-74.
67. Keller JN, Hanni KB, Markesbery WR. Possible involvement of proteasome inhibition in aging: implications for oxidative stress. Mechanisms of ageing and development. 2000;113(1):61-70.
68. Li F, Zhang L, Craddock J, Bruce-Keller AJ, Dasuri K, Nguyen A, Keller JN. Aging and dietary restriction effects on ubiquitination, sumoylation, and the proteasome in the heart. Mechanisms of ageing and development. 2008;129(9):515-21.
69. Bulteau AL, Szweda LI, Friguet B. Age-dependent declines in proteasome activity in the heart. Archives of biochemistry and biophysics. 2002;397(2):298-304.
70. Inuzuka Y, Okuda J, Kawashima T, Kato T, Niizuma S, Tamaki Y, Iwanaga Y, Yoshida Y, Kosugi R, Watanabe-Maeda K, Machida Y, Tsuji S, Aburatani H, Izumi T, Kita T, Shioi T. Suppression of phosphoinositide 3-kinase prevents cardiac aging in mice. Circulation. 2009;120(17):1695-703.
71. Wohlgemuth SE, Julian D, Akin DE, Fried J, Toscano K, Leeuwenburgh C, Dunn WA, Jr. Autophagy in the heart and liver during normal aging and calorie restriction. Rejuvenation research. 2007;10(3):281-92.
72. Kubli DA, Quinsay MN, Gustafsson AB. Parkin deficiency results in accumulation of abnormal mitochondria in aging myocytes. Communicative & integrative biology. 2013;6(4):e24511.
73. He C, Bassik MC, Moresi V, Sun K, Wei Y, Zou Z, An Z, Loh J, Fisher J, Sun Q, Korsmeyer S, Packer M, May HI, Hill JA, Virgin HW, Gilpin C, Xiao G, Bassel-Duby R, Scherer PE, Levine B. Exercise-induced BCL2-regulated autophagy is required for muscle glucose homeostasis. Nature. 2012;481(7382):511-5.
74. Wilson MG, Ellison GM, Cable NT. Basic science behind the cardiovascular benefits of exercise. Heart (British Cardiac Society). 2015;101(10):758-65.
75. Gurusamy N, Lekli I, Mukherjee S, Ray D, Ahsan MK, Gherghiceanu M, Popescu LM, Das DK. Cardioprotection by resveratrol: a novel mechanism via autophagy involving the mTORC2 pathway. Cardiovascular research. 2010;86(1):103-12.
76. Flynn JM, O'Leary MN, Zambataro CA, Academia EC, Presley MP, Garrett BJ, Zykovich A, Mooney SD, Strong R, Rosen CJ, Kapahi P, Nelson MD, Kennedy BK, Melov S. Late-life rapamycin treatment reverses age-related heart dysfunction. Aging cell. 2013;12(5):851-62.
77. Wilkinson JE, Burmeister L, Brooks SV, Chan CC, Friedline S, Harrison DE, Hejtmancik JF, Nadon N, Strong R, Wood LK, Woodward MA, Miller RA. Rapamycin slows aging in mice. Aging cell. 2012;11(4):675-82.

78. Adao R, de Keulenaer G, Leite-Moreira A, Bras-Silva C. Cardiotoxicity associated with cancer therapy: pathophysiology and prevention strategies. Revista portuguesa de cardiologia : orgao oficial da Sociedade Portuguesa de Cardiologia = Portuguese journal of cardiology : an official journal of the Portuguese Society of Cardiology. 2013;32(5):395-409.
79. Dazzi H, Kaufmann K, Follath F. Anthracycline-induced acute cardiotoxicity in adults treated for leukaemia. Analysis of the clinico-pathological aspects of documented acute anthracycline-induced cardiotoxicity in patients treated for acute leukaemia at the University Hospital of Zurich, Switzerland, between 1990 and 1996. Annals of oncology : official journal of the European Society for Medical Oncology. 2001;12(7):963-6.
80. Lipshultz SE, Lipsitz SR, Sallan SE, Dalton VM, Mone SM, Gelber RD, Colan SD. Chronic progressive cardiac dysfunction years after doxorubicin therapy for childhood acute lymphoblastic leukemia. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2005;23(12):2629-36.
81. Sterba M, Popelova O, Lenco J, Fucikova A, Brcakova E, Mazurova Y, Jirkovsky E, Simunek T, Adamcova M, Micuda S, Stulik J, Gersl V. Proteomic insights into chronic anthracycline cardiotoxicity. Journal of molecular and cellular cardiology. 2011;50(5):849-62.
82. Fan GC, Zhou X, Wang X, Song G, Qian J, Nicolaou P, Chen G, Ren X, Kranias EG. Heat shock protein 20 interacting with phosphorylated Akt reduces doxorubicin-triggered oxidative stress and cardiotoxicity. Circulation research. 2008;103(11):1270-9.
83. Naka KK, Vezyraki P, Kalaitzakis A, Zerikiotis S, Michalis L, Angelidis C. Hsp70 regulates the doxorubicin-mediated heart failure in Hsp70-transgenic mice. Cell stress & chaperones. 2014;19(6):853-64.
84. Kiyomiya K, Matsuo S, Kurebe M. Proteasome is a carrier to translocate doxorubicin from cytoplasm into nucleus. Life sciences. 1998;62(20):1853-60.
85. Sishi BJ, Loos B, van Rooyen J, Engelbrecht AM. Doxorubicin induces protein ubiquitination and inhibits proteasome activity during cardiotoxicity. Toxicology. 2013;309:23-9.
86. Fekete MR, McBride WH, Pajonk F. Anthracyclines, proteasome activity and multi-drug-resistance. BMC cancer. 2005;5:114.
87. Liu J, Zheng H, Tang M, Ryu YC, Wang X. A therapeutic dose of doxorubicin activates ubiquitin-proteasome system-mediated proteolysis by acting on both the ubiquitination apparatus and proteasome. American journal of physiology Heart and circulatory physiology. 2008;295(6):H2541-50.
88. Aryal B, Jeong J, Rao VA. Doxorubicin-induced carbonylation and degradation of cardiac myosin binding protein C promote cardiotoxicity. Proceedings of the National Academy of Sciences of the United States of America. 2014;111(5):2011-6.
89. Kobayashi S, Volden P, Timm D, Mao K, Xu X, Liang Q. Transcription factor GATA4 inhibits doxorubicin-induced autophagy and cardiomyocyte death. The Journal of biological chemistry. 2010;285(1):793-804.
90. Li DL, Wang ZV, Ding G, Tan W, Luo X, Criollo A, Xie M, Jiang N, May H, Kyrychenko V, Schneider JW, Gillette TG, Hill JA. Doxorubicin Blocks Cardiomyocyte Autophagic Flux by Inhibiting Lysosome Acidification. Circulation. 2016;133(17):1668-87.
91. Pizarro M, Troncoso R, Martinez GJ, Chiong M, Castro PF, Lavandero S. Basal autophagy protects cardiomyocytes from doxorubicin-induced toxicity. Toxicology. 2016;370:41-8.
92. Bartlett JJ, Trivedi PC, Yeung P, Kienesberger PC, Pulinilkunnil T. Doxorubicin impairs cardiomyocyte viability by suppressing transcription factor EB expression and disrupting autophagy. The Biochemical journal. 2016;473(21):3769-89.
93. Bartlett JJ, Trivedi PC, Pulinilkunnil T. Autophagic dysregulation in doxorubicin cardiomyopathy. Journal of molecular and cellular cardiology. 2017;104:1-8.
94. Weiner A, Kaminaris A, Kobayashi S, Gerdes M, Liang Q. The Role of Mitophagy in Doxorubicin-Induced Cardiomyocyte Death. The FASEB Journal. 2016;30(1 Supplement):1015.2.
95. Mahler HC, Friess W, Grauschopf U, Kiese S. Protein aggregation: pathways, induction factors and analysis. Journal of pharmaceutical sciences. 2009;98(9):2909-34.
96. Kushnirov VV, Alexandrov IM, Mitkevich OV, Shkundina IS, Ter-Avanesyan MD. Purification and analysis of prion and amyloid aggregates. Methods (San Diego, Calif). 2006;39(1):50-5.
97. Nizhnikov AA, Alexandrov AI, Ryzhova TA, Mitkevich OV, Dergalev AA, Ter-Avanesyan MD, Galkin AP. Proteomic screening for amyloid proteins. PLoS One. 2014;9(12):e116003.
98. Tao H, Liu W, Simmons BN, Harris HK, Cox TC, Massiah MA. Purifying natively folded proteins from inclusion bodies using sarkosyl, Triton X-100, and CHAPS. BioTechniques. 2010;48(1):61-4.

99. Choksawangkarn W, Edwards N, Wang Y, Gutierrez P, Fenselau C. Comparative study of workflows optimized for in-gel, in-solution, and on-filter proteolysis in the analysis of plasma membrane proteins. Journal of proteome research. 2012;11(5):3030-4.
100. Feist P, Hummon AB. Proteomic challenges: sample preparation techniques for microgram-quantity protein analysis from biological samples. International journal of molecular sciences. 2015;16(2):3537-63.
101. Arnolda L, McGrath B, Cocks M, Sumithran E, Johnston C. Adriamycin cardiomyopathy in the rabbit: an animal model of low output cardiac failure with activation of vasoconstrictor mechanisms. Cardiovascular research. 1985;19(6):378-82.
102. de Groot NS, Aviles FX, Vendrell J, Ventura S. Mutagenesis of the central hydrophobic cluster in Abeta42 Alzheimer's peptide. Side-chain properties correlate with aggregation propensities. The FEBS journal. 2006;273(3):658-68.
103. Fic E, Kedracka-Krok S, Jankowska U, Pirog A, Dziedzicka-Wasylewska M. Comparison of protein precipitation methods for various rat brain structures prior to proteomic analysis. Electrophoresis. 2010;31(21):3573-9.
104. Manadas BJ, Vougas K, Fountoulakis M, Duarte CB. Sample sonication after trichloroacetic acid precipitation increases protein recovery from cultured hippocampal neurons, and improves resolution and reproducibility in two-dimensional gel electrophoresis. Electrophoresis. 2006;27(9):1825-31.
105. Liang X, Ubhayakar S, Liederer BM, Dean B, Ran-Ran Qin A, Shahidi-Latham S, Deng Y. Evaluation of homogenization techniques for the preparation of mouse tissue samples to support drug discovery. Bioanalysis. 2011;3(17):1923-33.
106. Tan NJ, Daim LD, Jamil AA, Mohtarrudin N, Thilakavathy K. An effective placental cotyledons proteins extraction method for 2D gel electrophoresis. Electrophoresis. 2017;38(5):633-44.
107. Aye TT, Scholten A, Taouatas N, Varro A, Van Veen TA, Vos MA, Heck AJ. Proteome-wide protein concentrations in the human heart. Molecular bioSystems. 2010;6(10):1917-27.
108. David DC, Ollikainen N, Trinidad JC, Cary MP, Burlingame AL, Kenyon C. Widespread protein aggregation as an inherent part of aging in C. elegans. PLoS biology. 2010;8(8):e1000450.
109. Andres AM, Stotland A, Queliconi BB, Gottlieb RA. A Time to Reap, a Time to Sow: Mitophagy and Biogenesis in Cardiac Pathophysiology. Journal of molecular and cellular cardiology. 2015;0:62-72.
110. Barton GP, Sepe JJ, McKiernan SH, Aiken JM, Diffee GM. Mitochondrial and Metabolic Gene Expression in the Aged Rat Heart. Frontiers in physiology. 2016;7:352.
111. Preston CC, Oberlin AS, Holmuhamedov EL, Gupta A, Sagar S, Syed RH, Siddiqui SA, Raghavakaimal S, Terzic A, Jahangir A. Aging-induced alterations in gene transcripts and functional activity of mitochondrial oxidative phosphorylation complexes in the heart. Mechanisms of ageing and development. 2008;129(6):304-12.
112. Rattan SI. Synthesis, modifications, and turnover of proteins during aging. Experimental gerontology. 1996;31(1-2):33-47.
113. Sachs HG, Colgan JA, Lazarus ML. Ultrastructure of the aging myocardium: a morphometric approach. The American journal of anatomy. 1977;150(1):63-71.
114. Fearnley CJ, Roderick HL, Bootman MD. Calcium signaling in cardiac myocytes. Cold Spring Harbor perspectives in biology. 2011;3(11):a004242.
115. Lucchesi PA, Sweadner KJ. Postnatal changes in Na,K-ATPase isoform expression in rat cardiac ventricle. Conservation of biphasic ouabain affinity. The Journal of biological chemistry. 1991;266(14):9327-31.
116. Nair RR, Nair P. Age-dependent variation in contractility of adult cardiac myocytes. The international journal of biochemistry & cell biology. 2001;33(2):119-25.
117. Maurel A, Hernandez C, Kunduzova O, Bompart G, Cambon C, Parini A, Frances B. Age-dependent increase in hydrogen peroxide production by cardiac monoamine oxidase A in rats. American journal of physiology Heart and circulatory physiology. 2003;284(4):H1460-7.
118. Santin Y, Sicard P, Vigneron F, Guilbeau-Frugier C, Dutaur M, Lairez O, Couderc B, Manni D, Korolchuk VI, Lezoualc'h F, Parini A, Mialet-Perez J. Oxidative Stress by Monoamine Oxidase-A Impairs Transcription Factor EB Activation and Autophagosome Clearance, Leading to Cardiomyocyte Necrosis and Heart Failure. Antioxidants & redox signaling. 2016;25(1):10-27.
119. Villeneuve C, Guilbeau-Frugier C, Sicard P, Lairez O, Ordener C, Duparc T, De Paulis D, Couderc B, Spreux-Varoquaux O, Tortosa F, Garnier A, Knauf C, Valet P, Borchi E, Nediani C, Gharib A, Ovize M, Delisle MB, Parini A, Mialet-Perez J. p53-PGC-1alpha pathway mediates oxidative mitochondrial damage and cardiomyocyte necrosis induced by monoamine oxidase-A upregulation: role in chronic left ventricular dysfunction in mice. Antioxidants & redox signaling. 2013;18(1):5-18.

120. Kabayama M, Sakoori K, Yamada K, Ornthanalai VG, Ota M, Morimura N, Katayama K, Murphy NP, Aruga J. Rines E3 ubiquitin ligase regulates MAO-A levels and emotional responses. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2013;33(32):12940-53.
121. Kachur TM, Pilgrim DB. Myosin assembly, maintenance and degradation in muscle: Role of the chaperone UNC-45 in myosin thick filament dynamics. International journal of molecular sciences. 2008;9(9):1863-75.
122. Srikakulam R, Winkelmann DA. Chaperone-mediated folding and assembly of myosin in striated muscle. Journal of cell science. 2004;117(Pt 4):641-52.
123. Solomon V, Goldberg AL. Importance of the ATP-ubiquitin-proteasome pathway in the degradation of soluble and myofibrillar proteins in rabbit muscle extracts. The Journal of biological chemistry. 1996;271(43):26690-7.
124. Willis MS, Schisler JC, Portbury AL, Patterson C. Build it up-Tear it down: protein quality control in the cardiac sarcomere. Cardiovascular research. 2009;81(3):439-48.
125. Tam S, Spiess C, Auyeung W, Joachimiak L, Chen B, Poirier MA, Frydman J. The chaperonin TRiC blocks a huntingtin sequence element that promotes the conformational switch to aggregation. Nature structural & molecular biology. 2009;16(12):1279-85.
126. Boyle AJ, Shih H, Hwang J, Ye J, Lee B, Zhang Y, Kwon D, Jun K, Zheng D, Sievers R, Angeli F, Yeghiazarians Y, Lee R. Cardiomyopathy of Aging in the Mammalian Heart is Characterized by Myocardial Hypertrophy, Fibrosis and a Predisposition Towards Cardiomyocyte Apoptosis and Autophagy. Experimental gerontology. 2011;46(7):549-59.
127. Sevlever D, Jiang P, Yen SH. Cathepsin D is the main lysosomal enzyme involved in the degradation of alpha-synuclein and generation of its carboxy-terminally truncated species. Biochemistry. 2008;47(36):9678-87.
128. Wu P, Yuan X, Li F, Zhang J, Zhu W, Wei M, Li J, Wang X. Myocardial Upregulation of Cathepsin D by Ischemic Heart Disease Promotes Autophagic Flux and Protects Against Cardiac Remodeling and Heart Failure. Circulation Heart failure. 2017;10(7).
129. Shults NV, Das D, Suzuki YJ. Major vault protein in cardiac and smooth muscle. Receptors & clinical investigation. 2016;3(2).
130. Eghbali M, Eghbali M, Robinson TF, Seifter S, Blumenfeld OO. Collagen accumulation in heart ventricles as a function of growth and aging. Cardiovascular research. 1989;23(8):723-9.
131. Chen SW, Tung YC, Jung SM, Chu Y, Lin PJ, Kao WW, Chu PH. Lumican-null mice are susceptible to aging and isoproterenol-induced myocardial fibrosis. Biochemical and biophysical research communications. 2017;482(4):1304-11.
132. Fabrizi C, Businaro R, Lauro GM, Fumagalli L. Role of alpha2-macroglobulin in regulating amyloid beta-protein neurotoxicity: protective or detrimental factor? Journal of neurochemistry. 2001;78(2):406-12.
133. Chatterjee K, Zhang J, Honbo N, Karliner JS. Doxorubicin Cardiomyopathy. Cardiology. 2010;115(2):155-62.
134. Hanna AD, Lam A, Tham S, Dulhunty AF, Beard NA. Adverse effects of doxorubicin and its metabolic product on cardiac RyR2 and SERCA2A. Molecular pharmacology. 2014;86(4):438-49.
135. Yano M, Yamamoto T, Ikemoto N, Matsuzaki M. Abnormal ryanodine receptor function in heart failure. Pharmacology & therapeutics. 2005;107(3):377-91.
136. Saeki K, Obi I, Ogiku N, Shigekawa M, Imagawa T, Matsumoto T. Doxorubicin directly binds to the cardiac-type ryanodine receptor. Life sciences. 2002;70(20):2377-89.
137. Kassaar O, McMahon SA, Thompson R, Botting CH, Naismith JH, Stewart AJ. Crystal structure of histidine-rich glycoprotein N2 domain reveals redox activity at an interdomain disulfide bridge: implications for angiogenic regulation. Blood. 2014;123(12):1948-55.
138. Poon IK, Patel KK, Davis DS, Parish CR, Hulett MD. Histidine-rich glycoprotein: the Swiss Army knife of mammalian plasma. Blood. 2011;117(7):2093-101.
139. Juarez JC, Guan X, Shipulina NV, Plunkett ML, Parry GC, Shaw DE, Zhang JC, Rabbani SA, McCrae KR, Mazar AP, Morgan WT, Donate F. Histidine-proline-rich glycoprotein has potent antiangiogenic activity mediated through the histidine-proline-rich domain. Cancer research. 2002;62(18):5344-50.
140. Hayashi K, Yamauchi K, Yamamoto N, Tsuchiya H, Tomita K, Amoh Y, Hoffman RM, Bouvet M. Dual-color imaging of angiogenesis and its inhibition in bone and soft tissue sarcoma. The Journal of surgical research. 2007;140(2):165-70.
141. Bujak M, Frangogiannis NG. The role of IL-1 in the pathogenesis of heart disease. Archivum immunologiae et therapiae experimentalis. 2009;57(3):165-76.

142. Frangogiannis NG. Interleukin-1 in cardiac injury, repair, and remodeling: pathophysiologic and translational concepts. Discoveries (Craiova, Romania). 2015;3(1).
143. Zhu J, Zhang J, Xiang D, Zhang Z, Zhang L, Wu M, Zhu S, Zhang R, Han W. Recombinant human interleukin-1 receptor antagonist protects mice against acute doxorubicin-induced cardiotoxicity. European journal of pharmacology. 2010;643(2-3):247-53.
144. Okada H, Lai NC, Kawaraguchi Y, Liao P, Copps J, Sugano Y, Okada-Maeda S, Banerjee I, Schilling JM, Gingras AR, Asfaw EK, Suarez J, Kang S-M, Perkins GA, Au CG, Israeli-Rosenberg S, Manso AM, Liu Z, Milner DJ, Kaufman SJ, Patel HH, Roth DM, Hammond HK, Taylor SS, Dillmann WH, Goldhaber JI, Ross RS. Integrins protect cardiomyocytes from ischemia/reperfusion injury. The Journal of Clinical Investigation. 2013;123(10):4294-308.

8. SUPPLEMENTARY DATA

8.1) Supplement A: Optical density in aging animal model

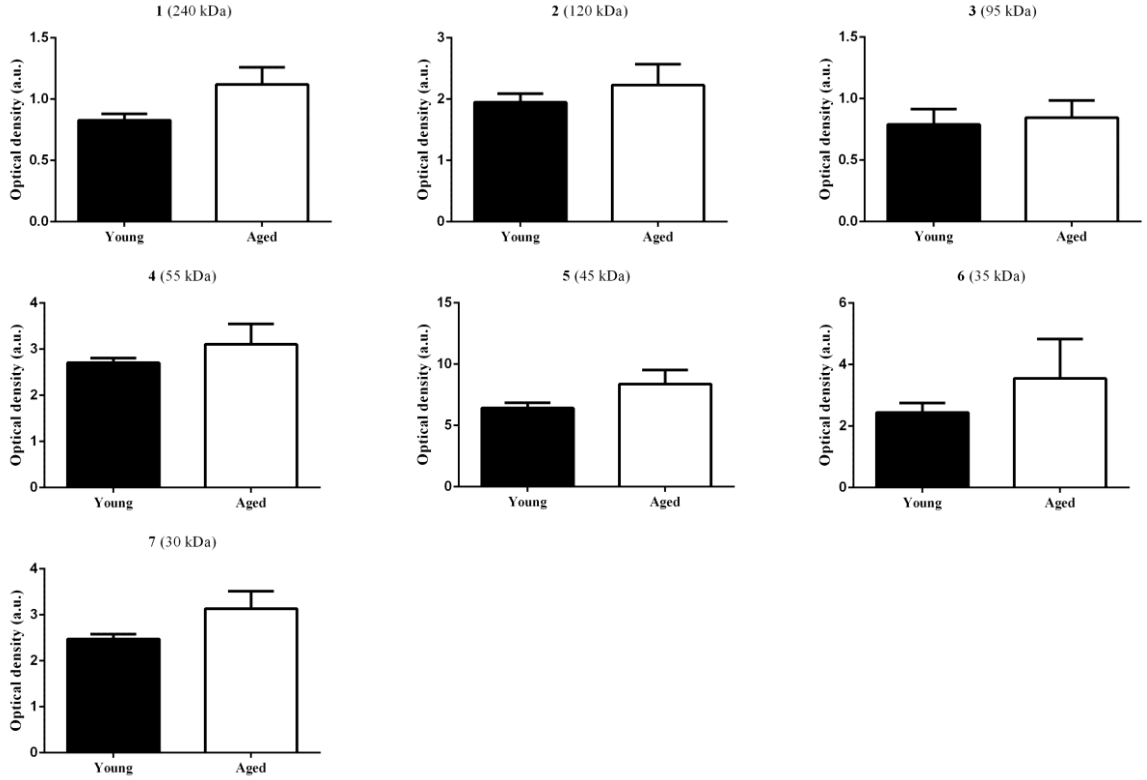

Figure 28: Optical density (in arbitrary units) comparison of the bands with estimated molecular weight of 240, 125, 95, 55, 45, 35 and 30 kDa between Young and Aged groups. No differences were observed [1) p=0.0870; 2) p=0.4687; 3) p=0.5185; 4) p=0.4013; 5) p=0.1504; 6) p=0.0972; 7) p=0.1273]. Values are represented by mean ± SEM. n=5 in each group.

8.2) Supplement B: Optical density in Doxo-induced cardiotoxicity animal model

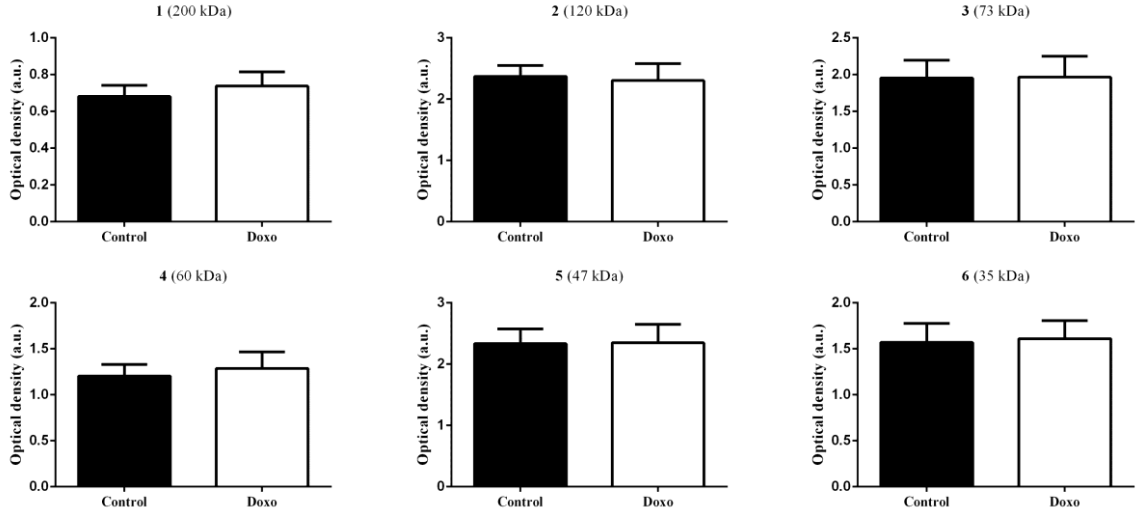

Figure 29: Optical density (in arbitrary units) comparison of the bands with estimated molecular weight of 200, 120, 73, 60, 47 and 35 kDa between Controls and Doxo groups. No differences were observed [1) p=0.5769; 2) p=0.8421; 3) p=0.9741; 4) p=0.7085; 5) p=0.9674; 6) p=0.8893]. Values are represented by mean ± SEM. n=5 in each group.

8.3) Supplement C: Cohen's d magnitude for proteins identified in aging animal model

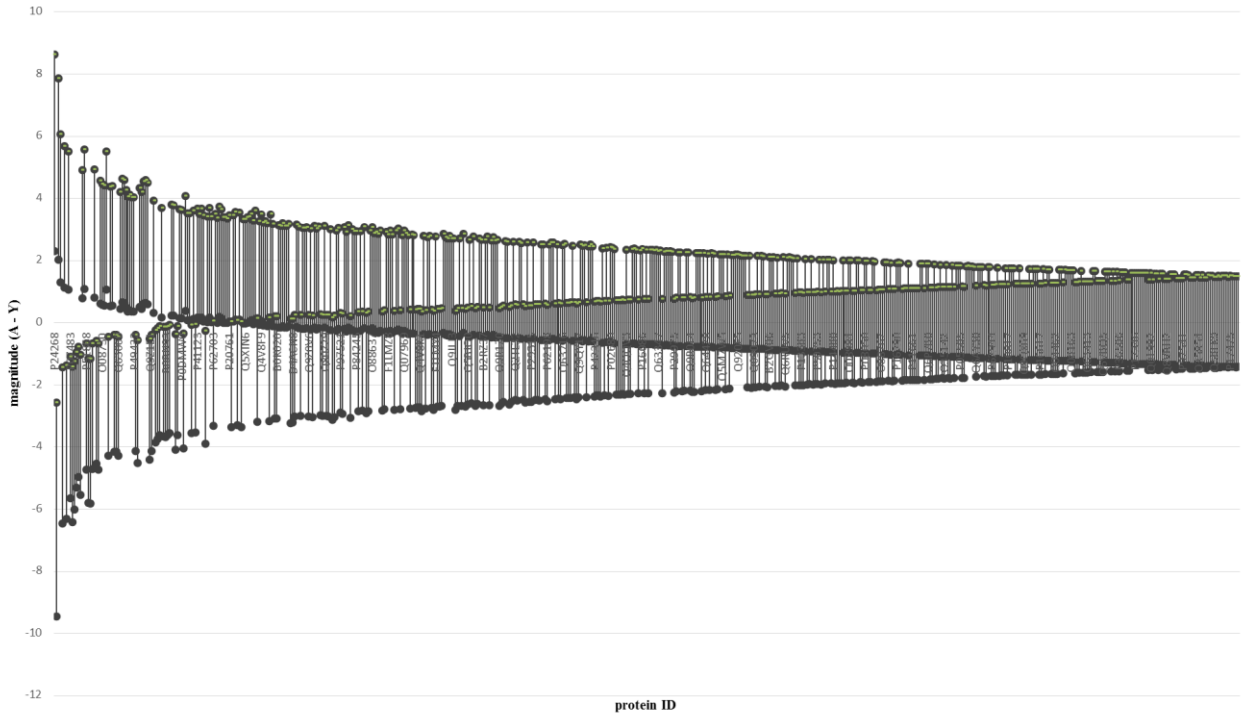

Figure 30: Representation of 95% confidence intervals of Cohen's d magnitude for each protein identified in all Young and Aged groups, simultaneously. Proteins with confidence intervals positives are increased in Aged group. Proteins with confidence intervals negatives are increased in Young group.

8.4) Supplement D: Cohen's d magnitude for proteins identified in Doxo-induced cardiotoxicity animal model

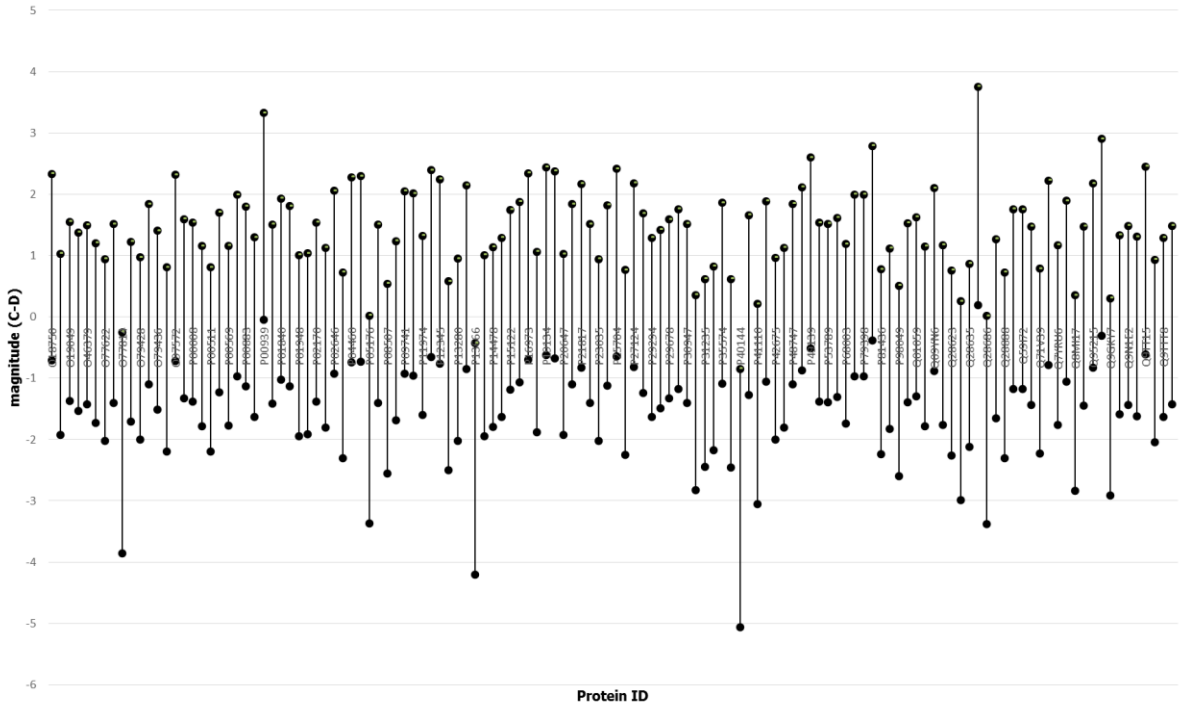

Figure 31: Representation of 95% confidence intervals of Cohen's d magnitude for each protein identified in all Control and Doxo groups, simultaneously. Proteins with confidence intervals positives are increased in Doxo group. Proteins with confidence intervals negatives are increased in Control group.